Cell Cycle Studies during Carcinogenesis in the Hamster Cheek Pouch.

by

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INTRODUCTION

Experimental Induction of Tumours:-

The first successful production of tumours by the topical application of a chemical carcinogen was by Yamagiwa and Ichikawa in 1915, one hundred and forty years after Sir Percival Pott described the relationship between soot and cancer of the scrotal skin in chimney sweeps. Yamagiwa and Ichikawa made repeated topical applications of coal tar on the ear skin of rabbits, two to three times a week, over periods up to one year or more. They noticed papillomatous new growths on the ears treated for 30 - 100 days, the number of new growths increasing with time. Carcinomatous change was observed between 55 and 360 days, and in most cases after 150 days from beginning of treatment. The tumours became invasive, in some cases, with regional lymph node metastasis, with subsequent emaciation and death of the animal.

Although similar experiments had been tried before, most of the earlier workers were unsuccessful either because they were unfortunate in choosing experimental animals that were resistant to carcinogenesis (e.g. Hanau, 1889; Cazin, 1894), or due to the fact that the painting was not continued long enough although the experimental animals were suitable (Bayon, 1912). After the report of Yamagiwa and Ichikawa, an enormous amount of research was undertaken in experimental tar carcinogenesis (reviewed by Woglom, 1924; Seeling & Cooper, 1933) and a further impetus was added by the synthesis of the first carcinogenic hydrocarbon 1:2:5:6 dibenzanthracene in a pure form by Kennaway and his colleagues, and the isolation of the carcinogenic hydrocarbon 3:4 benzpyrene from coal tar (Cook et al., 1933). From then onwards increasing numbers of pure chemical carcinogens became available.

The/

The availability of synthetic carcinogenic hydrocarbons opened a new era in the field of research into chemical carcinogenesis and a large number of workers all over the world have been investigating different aspects of the production of tumours by topical application of chemical carcinogens. As they are not relevant to the work to be described, neither the chemistry nor the mode of action of chemical carcinogens will be discussed here; suffice it to say that voluminous work has been done in this field (reviewed by Cook & Kennaway, 1938 and 1940; Haddow & Kon, 1947; Heiger, 1961; Boyland, 1964) and that certain chemical carcinogens, when applied topically, are capable of inducing carcinogenesis in the treated area of a suitable host.

Experimental Induction of Tumours on Oral Mucous Membrane:-

Although Bonne (1927) reported that he had produced one squamous cell carcinoma by tarring the mouths of 50 rats, subsequent attempts to induce experimental oral tumours by topical application of carcinogenic hydrocarbons (Levy, 1948; Levy et al., 1951) or tobacco smoke (Kreshover, 1952) proved to be unfruitful.

Salley (1954) was the first to be successful in producing experimental oral tumours by the topical application of carcinogenic hydrocarbons. He painted the cheek pouches of six groups of hamsters, each with 0.5% solutions of one of three carcinogenic hydrocarbons, viz. 9:10 dimethyl - 1:2 benzanthracene (DMBA), 20 - methylcholanthrene, and 3:4 benzpyrene, dissolved either in benzene or acetone, thrice per week, for 16 weeks with an additional period of observation of 9 weeks.

Tumours first appeared in the DMBA treated group after 7 weeks of painting. Animals painted with DMBA dissolved in benzene/

benzene showed a greater degree of inflammation and ulceration of the pouches during the initial stages of painting and a higher mortality rate than those painted with DMBA in acetone.

The latent periods for tumour formation in the benzpyrene - acetone group and the methylcholanthrene - acetone group were 16 and 25 weeks respectively. Only one-third of the animals painted with a benzene solution of methylcholanthrene, and none of those painted with benzpyrene in benzene, developed tumours. The incidence of tumours in the benzpyrene and methylcholanthrene groups proved to be less than that in the corresponding DMBA painted groups.

From these observations Salley concluded that an acetone solution of DMBA was the most satisfactory carcinogen for the induction of tumours in the hamster cheek pouch.

Although the animals painted with benzpyrene and methylcholanthrene developed tumours after much longer latent periods, the gross morphology of the tumours was similar to those in the DMBA painted animals.

Histologically, in all groups, four stages of development were seen: hyperplasia, benign papilloma formation, appearance of carcinoma in situ, and finally, squamous cell carcinoma with local invasion and metastases.

Since Salley's original work, tumours have been successfully induced in the palate, gingiva and tongue of hamsters (Salley & Kreshover, 1959; Al-Ani & Shklar, 1966; Dachi, 1967), palatal and buccal mucosa of rats (Wallenius, 1966) and the labial and lingual mucosa of mice (Chino et al., 1962; Fujino et al., 1965) by topical application of chemical carcinogens and a number of aspects of experimental oral carcinogenesis have been studied.

Although there has been dispute as to whether or not the lining of the hamster cheek pouch could be considered as part of the oral mucosa (Kolas, 1955; Goldhaber, 1957; Stormby & Wallenius, 1964), it has nevertheless proved to be an excellent site for a vast number of investigations in relation to experimental oral carcinogenesis.

Induction/

Induction of Experimental Tumours Using Tobacco and Betel Quid:-

As a causal relationship has been suggested between the habits of tobacco and betel chewing and pre-malignant and malignant lesions of the oral mucous membrane (Orr, 1933; Muir & Kirk, 1960; Pindborg et al., 1965), a number of attempts have been made to induce tumours in the oral mucosa of experimental animals with various forms of tobacco and betel quid.

Exposure of the labial mucosa of mice to tobacco smoke (Kreshover, 1952, 1955), the insertion of pastes of snuff or chewing tobacco into the hamster cheek pouch followed by the surgical isolation of the pouch (Peacock & Brawley, 1959; Peacock et al., 1960), and the exposure of the isolated monkey cheek pouch mucosa to Mainpuri tobacco (Cohen & Smith, 1966), all have failed to induce tumours.

Dunham & Herrold (1962) were unable to induce tumours in the hamster cheek pouch by implantation of pellets of beeswax containing various ingredients of betel quid (but not slaked lime). Implantation of similar pellets containing methylcholanthrene, however, resulted in a few squamous cell carcinomas.

At a later date Dunham et al. (1966) reported that repeated applications of calcium hydroxide in the hamster cheek pouch produced inflammatory and hyperplastic lesions, and a number of the hyperplastic pouches showed areas of epithelial atypia. They also stated that snuff was neither able to produce any lesions when applied alone, nor able to enhance the effects of calcium hydroxide when applied in conjunction with the latter.

Chang (1966) reported that, while application of extracts of either betel nut or lime to the hamster cheek pouch for periods up to 5 months only produced hyperplastic and hyperkeratotic lesions, treatment of the pouches with these two agents together produced a few papillomas.

It/

It was also interesting to note that Muir & Kirk (1960) were able to induce a few squamous cell carcinomas by painting the ear skin of mice with crude extracts of betel quid containing slaked lime for 2 years.

Sirsat & Kandarkar (1968), studying the effects of topical application of slaked lime on the buccal and palatal mucosa of rats, reported epithelial hyperplasia with acanthosis, hyperkeratosis and parakeratosis in animals painted up to one year. There was a marked hyalinisation of the connective tissue which, on electron microscopic examination, revealed a mild lateral swelling of the collagen fibrils together with an increase in the amount of amorphous material. There was no loss in the 640 Å periodicity of the fibrils. The tissue response was more pronounced in animals maintained on protein or Vitamin B deficient diets and in those treated with a corticosteroid preparation.

Although slaked lime appeared to play an important role in bringing about tissue changes in the above experiments, this does not exclude the possibility of a carcinogenic effect of the other ingredients of betel quid. The lack of success that has been met with in the induction of tumours by using these substances may be attributed to a low carcinogenic potency of these substances which requires long exposure periods before tumours could develop, and this is often limited by the short life span of the experimental animals.

Pathological Changes in the Hamster Cheek Pouch During Experimental Carcinogenesis:-

Salley (1957) described the gross and histological changes in the hamster cheek pouch during carcinogenesis by topical application of an 0.5% solution of DMBA in mineral oil, by serial killings of the animals.

Gross/

Gross Changes:-

Gross observations of the treated pouches showed evidence of inflammation and oedema during the first week, with raised whitish patches developing after 3 weeks of painting. The first evidence of tumour was seen after 15 applications of the carcinogen, i.e. after 5 weeks, the initial lesion consisting of a small raised nodule. Thereafter the pouches developed papillomatous outgrowths, some of them reaching 1 cm. in diameter.

Changes Revealed by Light Microscopy:-

Histologically, changes were seen in the pouches 24 hours after the first application of the carcinogen, characterised by acute inflammation with presence of inflammatory cells in the epithelium, as well as in the corium and in the underlying layers of striated muscle. In some places there was destruction of the epithelium leaving an ulcer.

After the first week of painting the inflammatory reaction began to subside in intensity, especially in the submucosal connective tissue and the muscle layer. Although Salley mentioned that the predominant features observed at this stage were degeneration and necrosis, a description of the type of degeneration or necrosis is not given in the text and the illustration used to demonstrate these changes shows merely masses of debris and/or slough on the surface of the slightly inflamed cheek pouch which is devoid of its epithelial covering in places.

With continued paintings there was an apparent regeneration of the epithelium, with marked hyperplasia and increased keratinisation observable from the third week onwards. The basal cells, which are normally arranged in a straight line on the connective tissue, began to develop rete-pegs and were hyperchromatic. The sub-mucosal connective/

connective tissue appeared to be thickened, showed an increased vascularity and evidence of proliferative activity.

The initial tumours that developed after 5 weeks of painting were papillomatous outgrowths of epithelium that did not invade the connective tissue. These tumours showed some cellular atypia with presence of prominent nucleoli and hyperchromatism. There was also evidence of increased vascularity at the base of these growths.

Tumours excised 7½ weeks after the beginning of painting showed carcinoma in situ with marked cellular atypia, loss of stratification, and presence of epithelial pearls, but without any local invasion of the connective tissue. The connective tissue itself showed scattered foci of chronic inflammatory cells and consisted of loose irregularly arranged fibres. Many sinusoid-like structures containing blood elements were also seen.

By the ninth week the histological picture of the tumours was one of squamous cell carcinoma with marked invasion of the underlying connective tissue. No metastatic deposits in the lymph nodes were seen in this series, although in a previous study Salley (1954) described lymph node metastasis in DMBA induced tumours observed over a period of 18 weeks from the development of initial tumours. The failure to observe any lymph node metastasis in his present study is probably a result of its shorter duration.

The gross and histological observations of Salley (1957) have been confirmed by a number of workers (e.g. Morris, 1957; Randall et al., 1964; Camilleri & Smith, 1965; Levij et al., 1967). However, the latent period for induction of tumours and the time taken for the development of the various intermediate stages during carcinogenesis vary from one report to another, apparently as a result of the different solvents used to dissolve the carcinogen (DMBA), and probably also due to the effect of the genetic background of the experimental animals.

Changes/

Changes Revealed by Electron Microscopy:-

Listgarten et al. (1963) investigated the ultrastructural changes in the epithelium of the hamster cheek pouch in response to applications of DMBA, xylene and mineral oil.

The DMBA group was painted thrice per week for 5 weeks, while the xylene controls were painted thrice per week for only 2 weeks. Animals in the mineral oil group were painted only twice, the second painting being 48 hours after the first.

Light microscopic observations in the DMBA treated animals showed stages of inflammation, ulceration and regeneration similar to those described by Salley (1957). The xylene treated pouches too showed a similar pattern of changes but occurring at a faster pace.

Listgarten et al. stated that the ultrastructural alterations seen in DMBA and xylene painted pouches were essentially the same within the period of the experiment. A marked widening of the intercellular spaces occurred during the first 2 weeks of painting in both DMBA and xylene treated pouches. The widening of intercellular spaces was recognisable as early as 24 hours following the first painting with presence of polymorphonuclear leukocytes and erythrocytes in them. Desmosomal junctions were frequently found to be stretched and often appeared to have become disrupted. There appeared to be an increase in the convolution of the cell surface with cytoplasmic processes extending into the patent intercellular spaces. Other less consistent findings in the DMBA and xylene treated groups were the relative abundance of rough surfaced endoplasmic reticulum when compared with the mineral oil treated group and the wide variation in the number of mitochondria present from cell to cell. Mineral oil treated pouches showed only mild changes with a slight increase in the intercellular spacing. However, a strict comparison cannot be made with the rest of the groups in view of the fact that only two paintings were made in this group.

Widening/

Widening of intercellular spaces with disruption of desmosomal junctions and an increase in the cell surface convolution were also described by Setala et al. (1960) in the interfollicular epidermis of mice after DMBA and methylcholanthrene applications.

In another experiment Listgarten et al. studied the ultrastructural characteristics of squamous cell carcinomas of the hamster cheek pouch induced by topical application of DMBA. The material examined consisted of well differentiated squamous cell carcinomas. The most striking feature in the majority of tumour cells was the clumping of what appeared to be bundles of tonofibrils characteristically arranged near the periphery of the cell. The tonofibrillar-clumps were seen to aggregate into larger masses in the vicinity of the epithelial pearls. The epithelial pearls themselves were composed of dense heterogenous masses resembling keratin and were surrounded by concentrically arranged epithelial cells containing clusters of tonofibrils and keratohyalin granules.

The nucleo-cytoplasmic ratio was independent of cell size and was found to be larger than that of normal cells or cells from inflamed pouches. The cytoplasm formed a relatively narrow band around the nucleus and the nucleoli were more prominent than those of control specimens.

The tumour cells at the epithelio-connective tissue interface presented a more irregular basal border than normal basal cells which made it difficult to visualise the basement membrane. However, in places the basement membrane appeared to be discontinuous. This was confirmed by the observations of Smith & Woods (1968) who reported discontinuities of the lamina densa of the basement membrane during experimental carcinogenesis of the hamster cheek pouch. In some places cytoplasmic processes of the basal cells were seen to extend into connective tissue through these gaps. Similar changes were also observed by Smith & Woods in some potentially malignant human oral leukoplakic lesions.

Changes/

Changes in the Pattern of Vasculature During the Induction of Tumours by Chemical Carcinogens:-

Rijhsinghani et al. (1968) reported on the vascular abnormalities produced in the hamster cheek pouch during the induction of tumours by topical application of 0.5% solution of benzpyrene in acetone. The carcinogen solution was painted 2 - 3 times a week, for 44 weeks. The vascular pattern of the treated pouches was studied in vivo at regular intervals after the beginning of painting by using the Sanders-Shubik chamber, and the animals were sacrificed following this for histological study of the lesions. Control animals painted with the solvent alone were also studied in a similar manner.

The vascular pattern of the normal cheek pouch consisted of vessels with uniform branching and a progressive diminution in calibre as they joined the sub-epithelial capillary network. Similar observations were made by Greenblatt & Shubik (1967).

Correlation of the observations on the vascular pattern with the histopathology of the lesions by Rijhsinghani et al. showed that there was a progressive increase in vascular abnormalities beginning at the stage of epithelial hyperplasia.

At the stages of inflammation and epithelial hyperplasia (15 weeks) the vascular changes were characterised by generalised hyperaemia, marked dilation and tortuosity of large veins, with the presence of many capillary-sized blood vessels in areas of ulceration. As the epithelial hyperplasia became more marked, a few of the treated pouches exhibited vascular abnormalities in the form of loop formation and glomeruloid alterations.

During the stage of epithelial hyperplasia with cellular atypia there were additional changes affecting the contour of the veins giving them a beaded appearance. There was a greater amount of capillary neovascularisation at this stage than during the early stages of hyperplasia. One-third of the animals examined also showed saccular and fusiform aneurysms of the arteries.

With/

With the development of carcinoma in situ and squamous cell carcinoma the alterations in the venous walls became more pronounced, with successive fusiform dilations giving them a "linked sausage" appearance. There was a greater amount of neovascularisation, frequently showing a spongy pattern. Histological examination of the specimens showing spongy neovascularisation suggested that these were small blood filled spaces in the sub-epithelial connective tissue.

Control animals painted with acetone showed only slight epithelial hyperplasia with minor vascular changes consisting of dilation of vessels, loop formation and moderate venous tortuosity.

Although vascular abnormalities have also been reported in relation to hydrocarbon carcinogenesis of skin (Kreyberg, 1927; Korn & Urbach, 1966) and in relation to human tumours (Urbach & Graham, 1962; Kolstad, 1965), it is not clear whether these vascular changes are primary, due to a direct effect of the carcinogenic agent on the connective tissue, or they are secondary to the epithelial changes.

Studies on Exfoliative Cytology During Experimental Carcinogenesis:-

With increasing interest in exfoliative cytology as a diagnostic aid in the early detection of oral malignancies, the hamster cheek pouch has become a favourite site for investigations on cytological changes during experimental carcinogenesis, and their correlation to the histopathological observations.

Stahl (1963a & b) was the first to report on the cytological changes at various stages during the induction of tumours in the hamster cheek pouch and relate them to the histological appearance of the lesions. Tumours were induced by the topical application of a solution of 0.5% DMBA in mineral oil, thrice per week, for 18 weeks. The smears for cytology were obtained twice per week, prior to the sacrifice of the animal for histological/

histological study of the lesions. No control animals were used in this study.

During the first week of painting the smears obtained showed essentially flat anucleate cells, with a few polygonal cells with pyknotic nuclei. The histological description of the epithelium at this stage was consistent with that of normal epithelium, without even the inflammatory changes that were commonly observed by other workers during the first week of painting.

During the second week smears consisted of cells with large nuclei, some of them demonstrating nuclear abnormalities described as dyskaryosis. The histological picture at this stage was one of slight epithelial hyperplasia with parakeratosis and an inflammatory reaction in the sub-epithelial tissues.

During the third week of painting the epithelium showed marked hyperplasia with loss of polarity of the cells in places, and the cytological studies showed large numbers of cells with hyperchromatic nuclei of varying sizes.

Papillomatous outgrowths were first observable after 4 weeks of painting, the smears at this stage consisting of single, as well as sheets of, nucleated cells with scanty cytoplasm, and hyperchromatic nuclei of varying sizes with prominent nucleoli. These appearances persisted in the smears until the ninth week.

From the tenth to eighteenth week from the beginning of the experiment, the tumours examined were squamous cell carcinomas with the accompanying smears showing cells with hyperchromatism, cellular pleomorphism, prominent nucleoli, chromatin clumping and an increase in the nucleo-cytoplasmic ratio.

From these findings Stahl concluded that the exfoliated cells seen in the smears revealed cellular atypia prior to clinical appearance of tumours and that there was a consistant correlation between diagnosis derived from histological examination and the cytological picture.

Although/

Although atypical cells were observed by Stahl prior to the appearance of tumours, and in fact from the second week of painting, it seems very doubtful whether it would be possible to predict the histology of the lesion from an examination of the desquamated cells except at the stage of frank carcinoma. In fact, Randal et al. (1964) obtaining similar results, concluded that examination of exfoliated cells did not permit a reliable diagnosis regarding the stage of the lesions as evaluated by histological criteria.

Fishman & Greene (1966), studying the cytological changes during chemical carcinogenesis in the hamster cheek pouch and correlating them with clinical and histological findings, observed atypical cells in the smears from the end of the second week of painting when clinical observations revealed only slight redness of the pouch mucosa. The degree of cellular atypism increased over the next few weeks and was rather advanced by 7 weeks when malignant tumours were seen during histological examination. They were unable to detect from the cytological appearances the stage at which the change from the reversible stage of inflammation and hyperplasia to irreversible malignant transformation took place.

The cytological changes described in later reports (Camilleri & Smith, 1964, 1965; Levij et al., 1967) are slightly different from that of Stahl (1963). Camilleri & Smith observed inflammatory cells in their smears in addition to the nucleated epithelial cells during the period corresponding to the stage of inflammation and ulceration seen in histological specimens. During the period of regeneration and hyperplasia that followed, the smears consisted of anucleate squames similar to those from the control pouches. With the development of papillomatous outgrowths the cytological picture was dominated by nucleated epithelial cells with a few inflammatory cells. The epithelial cells showed varying degrees of atypism, eventually showing evidence of malignancy.

Camilleri/

Camilleri & Smith observed a close correlation between the cytological picture and the histological appearance of the lesions at all stages during the induction of tumours.

Levij et al. (1967), however, found that while there was good agreement between the cytological picture and the histopathological appearance of the lesions during the periods of inflammation, hyperplasia and frank carcinoma, smears did not consistently show atypical cells during the stages of carcinoma in situ and in some cases of early squamous cell carcinomas. Levij et al. considered that this lack of correlation between cytology and histology was due to the hyperkeratotic or parakeratotic nature of the surface of these lesions yielding only keratin squames and parakeratotic cells in the smears. Negative findings also have been obtained from the cytological examination of keratotic premalignant lesions in humans (Shklar et al., 1968).

Chaudhry et al. (1967) reported the results of a quantitative study on the degree of association between cytological findings and histopathological diagnoses during experimental carcinogenesis, and on the degree of agreement between the cytological and histopathological diagnoses of two independant investigators.

Tumours were induced by topical application of an 0.5% solution of DMBA, thrice per week, for 21 weeks and the duration of the experiment was divided into three periods, each consisting of 7 weeks, for purposes of cytological and histopathological correlations. Cytological and histological observations were made by the serial killings of animals at weekly intervals.

The smears were diagnosed as normal, suspicious of malignancy or as definitely malignant, and the histopathological diagnoses were classified under three groups. The first group included lesions with epithelial hyperplasia and hyperkeratosis, the second group included lesions with dyskeratosis and carcinoma in situ, while squamous cell carcinomas were included in the third group.

Chaudhry/

Chaudhry et al. stated that there was 90% agreement between the cytological and histological diagnoses during the first 7 weeks of the experiment, but this agreement dropped to 78% during the second period of 7 weeks and was only 76% during the last 7 weeks of the experiment.

Examination of the tabulated results of Chaudhry et al., however, shows that the estimates of the degree of agreement between cytological and histological diagnoses were not based on the total number of smears and tissue specimens examined during each of the three periods of 7 weeks. While the number of lesions examined by cytology and histology during the first, second and the last third of the experimental period was 17, 27 and 30 respectively, the corresponding numbers used by them to estimate the degree of agreement between cytological and histological diagnoses were only 10, 23 and 17 respectively. Only those lesions that were diagnosed as normal or malignant by cytology or histology, which either showed complete agreement between the two diagnoses or a complete disagreement (i.e. one that was diagnosed as normal in cytology, found to be a malignant lesion in histology and vice versa) were included for the estimation of the degree of agreement between the two diagnoses.

For instance, out of the 17 smears examined during the first 7 weeks of the experiment, 9 were in agreement with the histological diagnosis, but all 5 of the smears reported as "suspicious of malignancy" turned out to be from benign lesions. Out of the 3 smears that showed definite signs of malignancy, 2 were found to be from dyskeratotic lesions and the third one from a benign lesion. From such results it is clear that the agreement between cytological and histological diagnoses would be less than the figure reported.

An/

An assessment of the degree of agreement between two investigators regarding cytological and histological diagnosis by Chaudhry et al. showed that there was a perfect agreement in 59% of the cases, with complete disagreement in only 7%.

There seems to be a wide variation in the degree of correlation between cytological and histopathological findings as observed by various workers during the induction of tumours in the hamster cheek pouch. The available evidence from these studies also strongly suggests that at present it is not possible to make a definite diagnosis regarding the histopathological nature of a lesion using exfoliative cytology alone.

Factors Influencing Tumour Induction in the Hamster Cheek Pouch

Age, Sex of Animal and Painting Routine:-

Morris (1957, 1961) investigated the effects of age and sex of the experimental animals, and the concentration, frequency, and method of application of the carcinogen on tumour production in the hamster cheek pouch.

When he painted the cheek pouches of 3 weeks, 6 weeks, 9 weeks and 18 month old hamsters with 0.5% DMBA in mineral oil, it was found that there was no significant difference in the latent period for tumour induction between animals of 3, 6 and 9 weeks. However, the latent period for tumour induction in the 18 months old animals was significantly higher than that for the rest of the groups. Morris concluded that younger animals were more susceptible to experimental carcinogenesis in the cheek pouch.

When/

When Morris painted groups of hamsters thrice per week, with one of 1.5%, 0.5%, 0.1% and 0.05% solution of DMBA in mineral oil, the animals painted with 1.5% solution of the carcinogen showed extreme reactions after the second painting with severe erythema and ulceration of the treated pouches. Some of the ulcerated pouches in this group never healed and half the experimental animals died before the development of any tumours. The 0.1% group exhibited only very mild changes during the initial stages of painting, while the 0.05% group was free of any observable changes. The latent period for the induction of tumours for the survivors in the 1.5% group was almost the same as that for animals painted with 0.5% solution of DMBA. Although all the animals in the 0.1% group developed tumours at the end of the experimental period of 20 weeks, the average latent period for tumour induction in this group was significantly higher than for the 0.5% and 1.5% groups. As the animals in the 0.05% group did not show any gross changes even after 15 weeks of treatment, the painting was discontinued at this stage. However, when the animals were sacrificed 5 weeks later, 6 out of 15 animals were found to have tumours, two of which were malignant. From these observations, it was concluded that 0.5% solution of DMBA was the most appropriate concentration for rapid induction of tumours, with maximum survival of animals.

When Morris tested the effect of the frequency of application of the carcinogen with twice weekly and thrice weekly paintings of the cheek pouch with 0.5% DMBA, the animals painted twice per week showed milder inflammatory reaction in the initial stages. By the ninth week from the beginning of treatment, tumours were present in all the animals painted thrice per week and only in 4 out of 15 animals painted twice per week. With continued painting all the animals developed tumours eventually. The latent period for tumour induction was found to be markedly shortened when the carcinogen was applied thrice weekly.

No/

No differences were observed in the survival rates or the response of male and female hamsters to the different regimens of application of the carcinogen in the above experiments.

Morris investigated two painting techniques, viz. the "dripping-brush" method, and "wiped-brush" method in which the brush was wiped once against the side of the container before painting. Both methods showed considerable uniformity in the amount of carcinogen delivered and a similar tissue response. However, the wiped-brush method was preferred because there was less likelihood of extra-oral contamination with this technique.

Effect of Nutrition:-

Animal experiments (reviewed by Tannenbaum & Silverstone, 1953), and epidemiological surveys (Wydner & Hoffmann, 1967) have shown that the nutritional state of the host plays an important role in its susceptibility to carcinogenesis. While factors like the caloric value of the diet, its composition and the proportion of the various components all seem to play a part, the degree of their effect on tumourigenesis does not seem to be similar for the various types of tumours at different sites.

Although the literature in relation to the effect of nutrition on carcinogenesis is extensive, little work has been done with a direct bearing on oral carcinogenesis.

Orr (1933) and Balendra (1949), while studying the incidence of oral carcinoma in betel chewers, observed a high prevalence of Vitamin A deficiency and mentioned the possibility of such a deficiency acting as a contributory factor in the genesis of tumours. Abels et al. (1942) reported on the relationship between Vitamin B complex deficiency and the incidence of lingual papillary atrophy and leukoplakia. Sirsat & Khanolkar (1960) found that the tissue changes aroused by the painting of capsaicin on the palatal and buccal mucosa of rats were more marked in animals deficient in Vitamin B complex and protein, than in the normal/

normal controls. Similar results were also obtained when the ear skin of normal and Vitamin A or B complex deficient mice were exposed to tobacco smoke (Kreshover, 1952). Although Kreshover made repeated applications of tobacco smoke on the lip mucosa of mice, no tissue changes were observed, probably due to the short duration of the experiments, considering the potency of the carcinogen and the comparative resistance of the oral mucous membrane to the induction of tumours by the topical application of chemical carcinogens.

In the hamster cheek pouch two series of investigations (Rowe & Gorlin, 1959; Salley et al., 1962) dealing with the effect of nutritional factors on tumour induction have been reported.

Rowe & Gorlin (1959) reported a higher incidence of tumours in Vitamin A deficient animals than in normal animals, when subjected to similar routines of carcinogen treatment. These animals were subjected to twice-weekly applications of DMBA for 13 weeks and were sacrificed after a further period of 7 weeks. Clinical and histological examinations of the treated pouches were carried out at this stage. Another group of animals treated with the carcinogen in a similar way, but fed on a restricted diet showed a lower incidence of tumours than those fed ad libitum or those fed on Vitamin A deficient diets, indicating that caloric restriction had a retarding effect on the process of carcinogenesis.

Salley et al (1962), studying the effect of chronic thiamine deficiency on carcinogenesis by topical application of a sub-optimal concentration (0.05%) of DMBA in the hamster cheek pouch, found that the latent period for tumour induction in the thiamine-deficient group was significantly shorter than that for the normal group, although the incidence of tumours was the same in both groups. The sub-optimal dose of the carcinogen/

carcinogen was used in this study in order to bring out any mild effect of chronic thiamine deficiency on tumour induction which would normally be masked by the use of strong concentrations of the carcinogen.

Although much work remains to be done in the field of nutrition and oral carcinogenesis, the observations of Rowe & Gorlin (1959), and Salley et al (1962) indicate that, while restriction of dietary intake inhibits carcinogenesis, the lack of an adequate intake of Vitamin A or thiamine has a facilitatory action on the process of carcinogenesis.

It is well established in other sites that the restriction of caloric intake has an inhibitary influence on carcinogenesis leading to a lower incidence and delayed development of a number of spontaneous and induced tumours in experimental animals (Tannenbaum & Silverstone, 1953). The amount of dietary restriction, as well as the composition of the restricted diet, appear to have an effect on the degree of the resulting tumour inhibition (Tannenbaum, 1945). The main inhibitory action of caloric restriction appears to occur at the stage of development and progression of the lesion, with little influence on the stage of tumour initiation itself (Tannenbaum, 1944).

Bullough & Eisa (1950), from their findings that the inhibition of epidermal mitotic activity in animals fed on low calorie diets was proportional to the degree of dietary restriction, suggested that the mechanism of tumour inhibition by caloric restriction was through its inhibitary effect on mitotic activity.

Effect of Solvents:-

Salley (1954) reporting the results of his studies on chemical carcinogenesis in the hamster cheek pouch, stated that acetone as the solvent for the carcinogen proved to be less toxic, with a lower mortality rate and slightly quicker induction of tumours than benzene. Salley's findings were in agreement with those of Bradbury et al. (1941) and Stowell & Cramer (1942) in relation to carcinogenesis of mouse skin.

At/

At a later date Salley (1955) reported that the use of mineral oil as the solvent for DMBA reduced the latent period for tumour development from 7 weeks (with DMBA in acetone) to 4½ weeks. Stormby & Wallenius (1964), however, claimed that mineral oil was prone to produce digestive disturbances.

Salley (1955) stated that direct examination of cheek pouches treated with DMBA in a volatile solvent (acetone) at varying intervals after application of the solution showed solid crystalline deposits of the carcinogen on the surface of the epithelium, while those painted with DMBA in mineral oil, showed the carcinogen solution in contact with the epithelium for longer periods of time because of the non-volatile nature of mineral oil.

Although Salley claimed that mineral oil acted as a co-carcinogen, the hastening of carcinogenesis by solvent-effects which increase penetration of the carcinogen thereby enhancing the effective tissue dosage cannot be considered as co-carcinogenic action (Brenblum, 1947; Salaman & Roe, 1964).

Dachi et al.(1967) reported that dimethyl sulfoxide, a substance with marked power of penetration through skin and mucous membranes, when used as the solvent for DMBA, produced significant reduction of the latent period for tumour induction in the hamster cheek pouch.

Comparison of the effects of dimethyl sulfoxide with that of mineral oil, as the solvent for DMBA showed that the former produced significant reduction of latent period for all the concentrations of the carcinogen tested. Neither the mean survival rate of the tumour bearing animals nor the morphological features of the tumours in the two groups were different from each other.

Dachi (1967) was also able to induce lingual tumours, with an average latent period of 94 days, by topical application of 0.5% DMBA in dimethyl sulfoxide. However, for purposes of comparison, there are no other reports at the present moment in which lingual tumours were induced using other solvents for DMBA.

Although/

Although the studies on experimental carcinogenesis in the hamster cheek pouch have shown that the solvents used influence the rapidity of tumour induction, the degree of the effect has been shown to be much more variable when the carcinogenic agent is injected sub-cutaneously than when topically applied (Wolf, 1952; Berenblum, 1954a).

Co-Carcinogenesis:-

Although the term "co-carcinogen" was introduced by Shear (1938) and co-carcinogenic action was defined as the augmentation of carcinogenesis by a non-carcinogenic agent when such an appropriate agent is applied concurrently with, or following a sub-optimal dose of the carcinogen (Berenblum, 1941), it was Deelman (1923) who first demonstrated such an effect.

Deelman claimed that scarification and incising of skin facilitated tar carcinogenesis and that the tumours which developed subsequently were localised close to the healing or healed wounds. These findings, sometimes referred to as the "Deelman effect", have been confirmed by a number of investigators (Pullinger, 1943; Friedewald & Rous, 1944; Riley & Pettigrew, 1945).

The outcome of the detailed study of the phenomenon of co-carcinogenesis has been the recognition of carcinogenesis as a step-wise process (Berenblum, 1941; Friedewald & Rous, 1944; Berenblum & Shukik, 1947) and the postulation of the two-stage hypothesis for the development of tumours. Although these conclusions were derived from studies of co-carcinogenesis in mouse skin, more recent work indicates that such mechanisms probably play a part in other sites too (Salaman & Roe, 1964).

According to the two-stage hypothesis the process of carcinogenesis consists of a phase of initiation during which the cells undergo an irreversible change to attain a latent neoplastic potential, followed by a phase of promotion during which these "latent neoplastic cells" progress to become tumours. The process of initiation is rapid and produces no visible/

visible changes in the appearance of the cells. Tumour promoters act slowly, and effective promotion requires long periods of time. The concepts of tumour initiation and promotion have been discussed by Berenblum (1954b; 1959), Walpole (1959), Setala (1961) and Salaman & Roe (1964).

A few tumour promoting substances originally thought to be non-carcinogenic, e.g. Croton oil, non-ionic surface active agents like Tween 60 etc., have recently been shown to be feebly carcinogenic yielding tumours after long periods of application (Roe, 1956; Ritchie, 1957; Della Porta et al., 1960). It is now suggested that the various agents cannot be separated into purely initiators or promotors (Salaman & Roe, 1964). Consequently, a tumour initiator has been defined as an agent which, when applied before or at the same time as a tumour promotor, produces many more tumours, or produces them more rapidly, than either agent does when applied alone or in a different order. A tumour promotor is an agent which has these effects when applied at the same time as, or after, a tumour initiator.

Although the finer aspects of initiation and promotion in tumour formation are still being discussed, the principle of the two-stage hypothesis remains widely accepted.

Although a number of studies have been carried out in relation to co-carcinogenesis in the oral mucous membrane, these studies appear to have been planned to recognise any possible co-carcinogenic action of the various agents used, rather than to elucidate the mecahnisms of initiation and promotion.

Croton Oil:-

Silberman & Shklar (1963) reported on their investigations of the effect of croton oil on chemical carcinogenesis in the cheek pouch of young and old hamsters. Croton oil was applied concurrently with the carcinogen (0.5% DMBA in mineral oil) in a concentration of 1%. The controls used consisted of pouches painted with the carcinogen, mineral oil or croton oil.

Siberman/

Siberman & Shklar (1963) observed severe inflammation, ulceration and suppuration in the pouches of young animals treated with croton oil and DMBA, together with a delay in the development of tumours in these pouches. In the older animals, however, they claimed that croton oil acted as a co-carcinogen reducing the latent period for development of tumours from 10 weeks with DMBA alone, to 8 weeks, together with histological evidence of a greater degree of malignancy.

Although croton oil is known to be an effective tumour promotor for mouse skin (Berenblum, 1941), the results of Siberman & Shklar (1963) are not convincing because of the very small number of animals used, especially in view of the marginal difference in the latent period for development of tumours in the two groups. Also, the co-carcinogenic effect of croton oil cannot be demonstrated effectively when it is applied concurrently with an optimal dose of a potent carcinogen which in itself has strong initiating and promoting effects.

Tween 60 & Tween 80:-

Dachi (1962) reported on the co-carcinogenic action of Tween 60 (Polyoxyethlene Sorbitan monostearate) during the induction of tumours in the hamster cheek pouch.

Dachi stated that while an optimal concentration of DMBA (0.5%) in Tween 60, painted until the appearance of tumours did not have any influence on tumour induction, the use of a sub-optimal concentration (0.2%) of DMBA in Tween 60 produced a significant reduction in the latent period for tumour induction. Control animals were painted with the corresponding concentrations of DMBA in mineral oil. In another experiment, animals were painted with an optimal concentration of DMBA, dissolved either in Tween 60 or in mineral oil, but were given only 15 applications of the carcinogen. When these animals were observed following the period of carcinogen treatment, it was found that the latent period for appearance of tumours in Tween 60 group was significantly shorter than that in the mineral oil group.

While/

While the morphological features of tumours in the two experimental groups were the same, the mean survival rate of the tumour bearing animals in the Tween 60 group was markedly shortened.

While the experiments of Dachi (1962) in which he used 0.2% DMBA, and a limited number of applications of 0.5% DMBA, effectively demonstrated the tumour promoting action of Tween 60, no enhancement of carcinogenesis was evident when 0.5% DMBA in Tween 60 was applied repeatedly until the appearance of tumours. This is mainly due to the masking of co-carcinogenic action when a tumour promoting agent is concurrently applied with an optimal concentration and dose of the carcinogen. The effective way of demonstrating co-carcinogenic action would be either by a concurrent application of the co-carcinogenic agent with a sub-optimal dose of the carcinogen, or by repeated applications of such an agent following a sub-optimal dose of the carcinogen alone, which may consist of one or more topical applications. The tumour promoting action of Tween 60 when repeatedly applied following a single application of a carcinogen has been demonstrated on mouse skin (Della Porta et al., 1960).

Sabes et al. (1959) reported that Tween 80 (Polyoxyethylene sorbitan mono-oleate) did not produce any augmentation of carcinogenesis in the hamster cheek pouch when applied concurrently with 0.5% DMBA. While all Tweens are not effective tumour promotors (Setala, 1961), it seems very doubtful whether a mild tumour promoting effect could be demonstrated by concurrently applying the particular agent with an optimal dose of the carcinogen.

Alcohol:-

Elzay (1966) reported on the local effect of alcohol on the induction of tumours with DMBA in the hamster cheek pouch. When the cheek pouches were painted with an 0.5% solution of DMBA either in mineral oil or alcohol, thrice per week, all the animals developed tumours eventually. Although a greater incidence/

incidence of tumours was observed in the DMBA alcohol group during the initial stages of the experiment, the average latent period for tumour induction in this group was only slightly shorter than that for the DMBA - mineral oil group. However, the DMBA alcohol group showed a slightly lower mortality rate during initial stages and higher survival rate of tumour bearing animals than the DMBA - mineral oil group.

Elzay (1966), studying the effect of repeated topical application of alcohol following a single application of 0.5% DMBA, stated that, although three of the nodular lesions that were observed during the experimental period had regressed by the time of sacrifice of the animals, histological examination showed carcinoma in situ in 13% of the pouches, while the pouches which received a single application of DMBA alone showed only hyperplastic changes.

Elzay (1966) concluded that alcohol acted as a promoting agent in experimental carcinogenesis of the hamster cheek pouch.

Henefer (1966), studying the effects of alcohol consumption on experimental induction of tumours in the hamster cheek pouch, was unable to observe any significant difference in the tumour incidence between hamsters that were receiving 30% ethanol as the only source of fluid intake and those which were drinking water instead.

The very small number of the tumour bearing animals in this experiment (4 in the experimental and 2 in the control group) does not permit a definite conclusion regarding the effects of alcohol consumption on experimental oral carcinogenesis.

Corticosteroids:-

Sabes et al (1959, 1960) reported that topical application of prednisolone acetate and cortisone acetate in conjunction with DMBA reduced the latent period for induction of tumours and increased the yield of tumours in the treated pouches.

The/

The effects of systemically administered cortisone on chemical induction of tumours in the hamster cheek pouch was studied by Shklar (1967) who reported a quicker induction of tumours together with a greater degree of malignancy in the cortisone treated animals.

Although the studies of Sabes et al. (1959, 1960) and Shklar (1967) indicate an augmentation of carcinogenesis by local and systemic administration of corticosteroids, the results of studies of the effect of these hormones on chemical carcinogenesis in other sites seems diverse (Sabes et al., 1963), and there does not seem to be a satisfactory explanation for this at the present moment.

Mechanical Irritation:-

Renstrup et al. (1962) reported their findings on the effect of mechanical irritation on carcinogenesis in the hamster cheek pouch. Mechanical irritation was provided by a stainless steel wire ligated around the lower first molar with its free end projecting into the cheek pouch and contacting its medial wall. Ulceration of the pouches with chronic mechanical irritation was evident 24 hours from the beginning of the experiment irrespective of whether or not they were treated with the carcinogen.

Tumours first appeared in pouches treated with carcinogen and mechanical irritation, after 4 weeks of treatment, and were confined to the edge of the ulcers. The group treated with the carcinogen alone developed tumours only after 10 weeks of painting. Although by the eighteenth week from the beginning of the experiment both groups showed similar incidence (100%) of tumours, the latent period for tumour induction was markedly reduced when the cheek pouches were chronically irritated.

Mechanical irritation alone or in conjunction with the vehicle did not produce any malignant change. Tissue changes produced in these groups were confined to inflammatory and ulcerative changes which persisted throughout the experimental period. When mechanical irritation alone was applied for 4 weeks and then followed up with carcinogen treatment, there

was/

was no significant change in the latent period for tumour induction.

Chino et al. (1962); Fujino et al. (1963), studying the effects of chronic mechanical irritation on carcinogenesis in the labial mucosa of mice, by topical application of 0.25% nitroquinoline N-oxide in propylene glycol, reported a higher incidence of tumours and a greater tendency to form metastatic deposits in the group that received the carcinogen in conjunction with mechanical irritation.

The studies on experimental oral carcinogenesis have demonstrated the co-carcinogenic action of local trauma, and are in agreement with the findings in other tissues (MacKenzie & Rous, 1941; Riley & Pettigrew, 1945).

Resistance of the Oral Mucous Membrane to the Induction of Experimental Tumours:

Even though the cheek pouch of the hamster responds readily to the topical application of chemical carcinogens, the oral mucous membrane of other experimental animals has been found to be refractory to the action of topically applied carcinogenic agents.

Levy (1948) was unable to produce malignant or pre-malignant changes by the topical application of 20-methyl-cholanthrene for 16 weeks on the labial gingiva of mice and hamsters.

Levy et al. (1951) compared the effects of a single application of DMBA in benzene on the mucous membrane and skin of the lip in mice. The only change observed in the mucous membrane of the treated animals sacrificed up to 32 days after the painting, was transient sub-mucous oedema during the first week. The epithelium itself did not show any pathological changes. On the other hand, the treated skin responded with inflammation and ulceration in the initial stages with evidence of regeneration and hyperplasia later on. From these observations Levy et al. concluded that the oral mucous membrane was refractory to the action of the carcinogen. They/

They stated that two factors were possibly responsible for this resistance, firstly the lack of a "portal of entry" for the carcinogen in the oral mucous membrane, due to the absence of sebaceous glands and/or, secondly the presence of a protective layer of saliva. They also suggested that the use of an anti-sialogogue or the extirpation of the major salivary glands in conjunction with the application of a carcinogen on the oral mucous membrane of experimental animals might help to clarify the problem.

Kreshover (1952) exposed the ear skin and the muco-cutaneous junction of the lower lip of mice, fed either on normal diet or on Vitamin B complex deficient diet, to tobacco smoke on alternate days for 76 days. The animals fed on normal diets showed only a slight redness and thickening of the treated ears at the end of the experimental period, while the animals in the Vitamin B complex deficient group showed marked changes with thickening and scarring of the ears as early as 20 days from the beginning of the treatment. Histological examination of the ears at the end of the experiment showed a mild epidermal hyperplasia with varying degrees of sub-cutaneous inflammation and fibrosis in the normal diet-group, and a marked epidermal hyperplasia with acanthosis and hyperkeratosis in the Vitamin B complex deficient group. The treated lip mucosa did not show any gross changes irrespective of whether the animals were fed on normal diet or Vitamin B complex deficient diets. Histologically the lip mucosa appeared normal except for parakeratosis seen in rare instances. Kreshover remarked that the observed difference in the response of the skin and mucous membrane to tobacco smoke applications could only partly be attributed to the basic differences in the morphology of these tissues and that the action of the tongue and saliva in removing the deposited irritant probably had a protective effect on the mucous membrane.

Kolas (1955) subjected the palatal mucosa of mice to three drops of 0.5% solution of methyl-cholanthrene, twice per week for periods ranging up to 6 months. During the process of instillation of the carcinogen solution on the palate there was inevitable/

inevitable contamination of the rest of the oral mucosa and the peri-oral skin. Neither the palate nor the rest of the oral mucous membrane showed any significant tissue changes after 6 months of treatment. However, squamous cell carcinomas were seen on the facial skin in 6 out of 24 animals.

From the observations of Levy (1948), Levy et al. (1951), Kreshover (1952) and Kolas (1955), it was generally accepted that the oral mucous membrane of most experimental animals had comparatively high resistance to the action of topically applied carcinogenic agents. In an attempt to investigate the nature of this resistance, essentially two lines of research were pursued; firstly, to find out whether saliva had any protective effect on the oral mucous membrane against the action of topically applied carcinogens, and secondly to clarify the need for a "portal of entry" before tumours could be induced by topical application of carcinogens.

Kolas (1955) was the first to investigate whether saliva had a protective anti-carcinogenic effect. He suggested that the susceptibility of the hamster cheek pouch to experimental induction of tumours was probably due to this tissue being subjected to a much lesser flow of saliva than is oral mucosa elsewhere. However, it has now been shown that even the mucous membrane of the oral cavity proper in hamsters is more susceptible to experimental carcinogenesis than those of other animals (Salley & Kreshover, 1959; Stormby & Wallenius, 1964; Wallenius, 1966).

In an attempt to demonstrate the possible anti-carcinogenic effect of saliva, if any, Kolas (1955) wetted the skin of both ears and the back of the neck in mice with human saliva twice per week and on the day following each application of saliva, 3 drops of 0.5% solution of 20-methylcholanthrene were applied to the saliva-treated area. Control animals received saline applications in place of saliva and were treated with the carcinogen in a similar manner. Although Kolas stated that all the animals in both control and experimental groups ultimately developed squamous cell carcinomas, the average latent/

latent period for development of tumours in the two groups was not mentioned. Kolas concluded that saliva did not possess an anti-carcinogenic property under the conditions of the experiment. These findings, however, do not rule out the possibility of a protective effect exerted by the physical cleansing action of saliva on the oral mucous membrane.

Kreshover & Salley (1957) extirpated the major salivary glands of hamsters and subjected the palatal mucosa to daily applications of whole tobacco smoke for 16 months. The oral tissues were free of any pathological changes at the end of the experiment. However, it should be noted that the carcinogen used in this study was of no significant potency and there was an appreciable amount of saliva present in the mouth as a result of secretions from the minor salivary glands.

Goldhaber et al. (1956) made applications of 0.3% solution of methylcholanthrene, for 3 months on the labial mucosa of mice after surgical removal of the major salivary and lacrimal glands. No pathological changes were seen in the labial mucosa of animals sacrificed during 8½ months. They concluded that the local action of saliva per se apparently does not explain the low susceptibility of the oral mucosa to chemical carcinogens. Goldhaber (1957), however, reported at a later date that in 3 out of 5 animals that were sacrificed 10 - 12 months after the initial treatment with the carcinogen, squamous cell carcinomas were seen in association with chronic ulcerations. He suggested that desalivation acted indirectly by producing stagnation of food and hair, leading to ulceration which acted as a portal of entry for the carcinogen or a co-carcinogen or both. He also stated that the ulceration which occurred during the initial stages of the application of the carcinogen in the hamster cheek pouch, may have contributed to the susceptibility of the cheek pouch mucosa to carcinogenesis.

Although/

Although it has been shown that the process of carcinogenesis is enhanced by the application of chronic trauma often leading to ulceration of the carcinogen treated area (Chino et al, 1962; Renstrup et al., 1962), ulceration of the painted area is not a pre-requisite for the chemical induction of tumours. Morris (1957) produced tumours in the hamster cheek pouch by applications of 0.05% solution of DMBA, a concentration that does not produce inflammatory or ulcerative changes. Also Salley & Kreshover (1959) did not observe any ulceration prior to tumour formation in the palates of hamsters painted with an 0.5% solution of DMBA.

As the degree of desalivation achieved in the experiments of Kreshover & Salley (1957) and Goldhaber et al. (1956) was only partial, and the carcinogen used in the former study was of no significant potency, their results cannot be considered as conclusive. Most of the evidence in support of a protective effect exerted by saliva comes from the series of experiments conducted by Wallenius.

Stormby & Wallenius (1964) painted the palate of hamsters with intact or reduced salivation (produced by surgical removal of the major salivary glands) with 0.5% solution of DMBA in acetone, thrice per week. Both groups of hamsters developed benign papillomas and squamous cell carcinomas after 4-6 months of painting. The total number of malignant and pre-malignant lesions in the desalivated group was significantly higher than those in the normal animals. However, although the number of animals that developed malignant tumours was higher in the desalivated group than in the group with intact salivation, the difference was not statistically significant. At a later date, Wallenius (1966) commented that it was the higher sensitivity of the oral mucosa of hamsters to carcinogenesis that masked the effect of reduced salivary secretion on tumour induction.

When/

When Wallenius (1965a) made tri-weekly applications of 0.5% solution of DMBA on the oral mucosa of rats, squamous cell carcinomas developed in a third of the treated animals after 16 months of painting. Similar treatment of the ear skin of rats invariably yielded squamous cell carcinomas after 6 months of treatment. When Wallenius (1965b) auto-transplanted the cheek skin of rats into the buccal mucosa and subjected the transplanted skin and the surrounding mucosa to DMBA paintings for 11 months, no changes were seen in the transplanted skin, while the surrounding mucosa showed slight epithelial hyperplasia and hyperkeratosis. Similar treatment of the cheek skin of unoperated animals invariably produced squamous cell carcinomas after 6 months of treatment. The transplanted skin showed persistent hair follicules and sebaceous glands even after 11 months of treatment. Wallenius concluded that the lack of sebaceous glands and hair follicules could not explain the low susceptibility of the oral mucous membrane to the action of carcinogens.

From the results of Hamner (1966) it now appears that it would have been preferable to have also studied the effects of the grafting procedure itself on the response to DMBA applications, by autografting the cheek skin of one side into the contralateral side. Hamner (1966) autografted discs of cheek pouch mucosa into the dorsal skin of hamsters and painted the autografted cheek pouch and the surrounding skin, thrice per week with an 0.5% solution of DMBA in mineral oil. Multiple papillomas developed on the painted area of the skin between the eighth and tenth week, while only one out of twenty animals developed a papilloma on the graft even after 12 weeks of painting. From these results Hamner concluded that the effect of the grafting itself may be one of the factors responsible for the non-response of the autografted cheek pouch.

Wallenius/

Wallenius (1966) reported the results of painting the buccal mucosa or the palate of normal and desalivated rats, thrice per week with an 0.5% solution of DMBA in acetone. Desalivation was achieved by surgical removal of the major salivary glands or by the repeated injections of the parasympatholytic drug methylscopolamine, or both. While the removal of major salivary glands only reduced the salivation to nine-tenths of the normal rate, a combination of surgical removal of major salivary glands and the repeated injection of methylscopolamine produced complete dryness of the oral mucosa. After 11 months of bilateral paintings on the buccal mucosa, Wallenius found that all the animals with dry mucosa developed squamous cell carcinomas, while none of the animals with normal salivation, and only one-third of the animals with reduced salivation, developed carcinomas. Animals painted on their palates showed similar results except that the incidence of tumours, after 11 months of treatment, was lower than that for the corresponding groups that received bilateral paintings on the buccal mucosa. Wallenius concluded that saliva exerted a protective action on the oral mucous membrane against the action of topically applied chemical carcinogens and that this protective effect was probably due to its presence on the surface of the mucous membrane as a moist mucous film.

These results were supported by his micro-fluorescent studies on frozen sections of oral mucosa of animals with normal salivation and those with dry mucosae, 1 to 72 hours after painting with a 1% solution of benzpyrene. Benzpyrene was used in preference to DMBA because of its more intense fluorescence. It was found that the fluorescence of the carcinogen was present in all layers of the epithelium of oral mucosa with inhibited salivation 3 hours after application of benzpyrene and persisted at least for 48 hours. Fluorescence was also observed in the sub-mucosa of these animals with increasing intensity from 16 hours to 24 hours. Animals with normal/

normal salivation showed fluorescence only in the superficial keratinised layers of the epithelium with no observable fluorescence in the deeper cellular layers or the sub-mucosa at any stage.

Although one cannot conclude from the absence of fluorescence that no carcinogen penetrated at all, as the amount of carcinogen penetrating may have been too minute to be detected by its fluorescence, these findings indicate that the presence of saliva reduces the amount of carcinogen penetrating into the oral mucous membrane, thereby making it more resistant to experimental induction of tumours by topical application of chemical carcinogens. Such protective properties have also been attributed to the presence of mucous in relation to experimental induction of tumours in the gastric mucosa (Ivy, 1945; Norden, 1957).

Although there exists reasonable evidence in support of a protective action of saliva, the position regarding the need for an anatomical portal of entry for experimental carcinogenesis is rather controversial, in spite of the fact that these two explanations need not be mutually exclusive.

Goldhaber et al. (1956) and Levy (1957, 1958, 1963) considered that the lack of a "portal of entry" for the carcinogen in the oral mucous membrane was important in relation to its resistance to experimental carcinogenesis.

However, their conclusions were drawn from the results of other workers (Simpson & Cramer, 1943; Lacassagne & Latarjet, 1946; Suntzeff et al, 1947) in relation to experimental carcinogenesis of the skin, with little and inconclusive evidence from studies on experimental oral carcinogenesis.

In their fluorescence microscopic studies Simpson & Cramer (1943) found that the carcinogenic hydrocarbons accumulated in the sebaceous glands of mouse skin immediately after a single topical application. Lacassagne & Latarjet (1946) reported that sebaceous glands and hair follicles played an important part in experimental carcinogenesis of the skin as a result of their findings that skin made devoid of these structures by scarring was resistant to induction of tumours by topical/

topical application of methylcholanthrene. Suntzeff et al. (1947) were unable to induce tumours by a single application of methylcholanthrene on the skin of new born swiss mice. No older controls were used in this experiment, although it was cited that Cramer & Stowell (1943) were successful in producing tumours in 43% of animals of 2½ to 3 months of age after similar treatment. Suntzeff et al. concluded that the lack of a portal of entry due to the rudimentary nature of the sebaceous glands and hair follicles in the skin of new born mice was responsible for its resistance to carcinogenesis. In the oral mucous membrane, Levy (1958) was successful in inducing lingual carcinomas in mice by sub-mucosal injection of an aquous suspension of methylcholanthrene. Although Levy claimed that his findings gave additional proof to the need for a portal of entry, his results are far from satisfactory as evidence in support of the "portal of entry hypotheses".

Although a number of claims have been made regarding the need for an anatomical portal of entry for the carcinogen, studies on the penetration of carcinogenic hydrocarbons after single and repeated topical applications in the cheek pouch, tongue and palate of hamsters using fluorescence microscopy (Salley, 1961), and the studies on the localisation of radioactivity, by autoradiography, after topical applications of ^{14}C labelled DMBA in the hamster cheek pouch (Meskin & Woolfrey, 1964), have shown that the carcinogen reaches the deeper layers of the epithelium and the sub-epithelial tissues after repeated topical applications. Listgarten et al. (1963) considered that the marked widening of intercellular spaces that occurred at the ultrastructural level as early as 24 hours after the first application of the carcinogen may facilitate passage of carcinogen into the deeper layers of the epithelium after repeated topical applications.

In addition to the findings of Salley (1961), Meskin & Woolfrey (1964) and Wallenius (1966) regarding penetration of carcinogenic hydrocarbons after topical applications, the fact that/

that it has been possible to induce carcinogenesis in the hamster cheek pouch, which does not possess any anatomical portal of entry, even in the absence of any surface ulcerations (Morris, 1957) challenges the absolute necessity for such a portal of entry.

Epithelio-connective Tissue Interaction During Carcinogenesis:-

There has been an increasing amount of evidence in recent years to indicate that the stroma plays an important role during the induction of tumours.

Orr (1934, 1935) from his observations that there was a high incidence of tumours in relation to scars and areas of reduced vascularity in humans, proceeded to test this using epidermal carcinogenesis in the mouse. Orr found that the local injection of adrenaline and scarification by insertion of suture threads produced an enhancement of tumour formation.

In a histological study of the changes produced by the topical application of carcinogenic and non-carcinogenic agents in the mouse skin, Orr (1938) observed that carcinogenic agents produced characteristic changes in the stroma and these changes developed more rapidly and pronouncedly with the more potent carcinogens. The stromal changes described included alteration of collagen into a fine-fibred, non-refractile mass, alteration of the texture of the elastic tissue which appeared to be deficient in places giving rise to "gaps", and a passive congestion of the subcutis. Orr (1938) also stated that there was a high incidence of tumours in relation to scars, and "gaps" in the elastic tissue of the subcutis. Non-carcinogenic agents, however, either produced no changes or produced an inflammatory response followed by increased cellular activity in the connective tissue leading to scar formation.

Orr considered that the stromal changes produced by carcinogenic agents played an important role in the neoplastic transformation of the epithelium.

More convincing evidence came from the experiments of Billingham et al. (1951) and Marchant & Orr (1953).

Billingham/

Billingham et al. (1951) reported that pure epidermal or thin split-skin grafts taken from a carcinogen treated area and implanted into a half-thickness bed on another part of the skin failed to develop tumours, while the treated area from which the grafts were taken subsequently re-epithelialised and showed a number of tumours. Tumours were also observed on grafts of untreated epidermis placed on the connective tissue bed of the area of skin that had been pre-treated with a carcinogen.

Marchant & Orr (1953), studying the effects of croton oil painting on epidermal grafts taken from a carcinogen treated area implanted into an untreated connective tissue bed, reported that there were no more tumours in the grafts than in animals that were painted with croton oil alone. The area from which the graft was taken showed many more tumours even without croton oil treatment.

While the results of Marchant & Orr (1953) give additional support to the suggestion that stroma plays an important role in carcinogenesis, they also contradict the concepts of initiation and promotion as proposed by Berenblum & Shubik (1947).

The epithelio-connective tissue interaction during carcinogenesis has also been demonstrated in viral carcinogenesis using tissue culture systems of salivary gland rudiments (Dawe et al., 1966) and tooth buds (Main, 1968).

Experiments in relation to the stromal influence on carcinogenesis by transplantation procedures similar to those of Billingham et al. (1951) and Marchant & Orr (1953) in the oral mucosa would prove interesting. The hamster cheek pouch would afford a suitable site, it being free of any accessory structures like hair follicles and sebaceous glands, as it has been suggested that there is a possibility that the tumours arising in relation to the carcinogen treated connective tissue may arise from proximal parts of the hair follicles that were left behind during the cutting of the graft (Billingham et al., 1951).

Hamner/

Hamner (1966) found that the cheek pouch mucosa autografted into dorsal skin in hamsters was more resistant to induction of tumours than the surrounding skin or the cheek pouch mucosa in situ. Hamner considered that the grafting procedure itself, or the influence of a different environment, may have produced this change. Billingham et al. (1951) considered the process of grafting enhanced tumour formation, while Marchant & Orr (1953) did not find any difference between the ultimate yield of tumours in areas of skin that were grafted and those which were unoperated and painted with a carcinogen. However, these results are not strictly comparable because in the experiments of Billingham et al. (1951) and Marchant & Orr (1953) the grafted skin was pre-treated with a carcinogen, while Hamner (1966) started the carcinogen treatment only 14 days after the grafting procedure. It would be profitable to extend the experiments of Hamner to clarify the effects of grafting procedure itself by switching grafts from one side of the cheek pouch to the other, and also study the epithelio-connective tissue interaction during chemical induction of tumours in the hamster cheek pouch by transplantation procedures.

Statement of Problems:-

The present investigation was planned to study the cell proliferation and cell cycle characteristics of the epithelium of the cheek pouch and palate in hamsters and thereafter the alterations in these parameters that occur during the induction of tumours in the cheek pouch by topical application of a chemical carcinogen.

Cell proliferation was studied using tritiated thymidine to estimate the duration of cell cycle and its phases, while mitotic rate was studied by the injection of a cytostatic drug. These two methods allow study of the cells at two different phases of their cell cycle.

Since the beginning of this work in 1966 there has been a publication in this field by Reiskin & Berry (1968) and their results will be discussed later.

CELL POPULATION KINETICS

The definition of a cell population depends on the problem under investigation and usually simply considered as the group of cells under discussion. A cell population may consist of one or more types of cells and when it is heterogenous it is convenient to sub-divide the population into a number of compartments. A cell compartment is one that would consist of a group of cells that could be distinguished from the rest of the cells of the population by means of functional, morphological or spatial characteristics (Quastler, 1963).

From the definitions of a cell population and cell compartment, it follows that a group of cells that constitute only one of the compartments in a particular study may, in fact, become the population in another, if the latter study was concerned only with that particular group of cells. However, once the extents and limits of these two are described they afford a useful way of defining and sub-dividing a cell population.

Classification of Cell Populations According to Their Proliferative Activity:-

The existence of cell renewal in certain tissues was first recognised by Bizzozero in 1892. He deduced that the presence of mitotic figures only in the crypts of the small intestine implied that the newly formed cells should migrate into the villi, become functional cells and ultimately be shed off.

Bizzozero (1894) recognising the variation in the number of mitotic figures in the different adult tissues, considered that the extent of the mitotic activity shown by the tissues was indicative of the degree of physiological regeneration that was taking place in them.

Bizzozero/

Bizzozero (1894) also classified the tissues of the body into three broad groups. The first group included tissues that showed mitotic activity throughout the life of the animal, e.g. epithelial coverings, blood forming tissues, etc., and this group of tissues was considered to be composed of transient elements (elementi labili). Tissues showing mitotic activity only up to the time of birth, or for a short time afterwards, were designated into the second group. This group included liver, kidney, pancreas, salivary glands, smooth muscle, bone, cartilage, and were described to be composed of stable elements (elementi stabili). Nervous tissue and striped muscle were considered to be composed of cells that had lost their capacity to divide at an early stage of embryonic life, and these tissues were described to be consisting of perennial elements (elementi perenni).

The popular classification of tissues that is used nowadays was put forward by Leblond et al. (1959) based on two parameters, viz. the number of cells of a tissue that incorporated tritiated thymidine, and the rate at which these labelled cells were lost from these tissues.

Leblond et al. (1959) gave a single injection of tritiated thymidine to 3-day old and adult mice and rats that were sacrificed within a few hours of the injection. The number of labelled cells in the various tissues were determined by autoradiography. In addition, a group of young animals (3-day old) were injected with tritiated thymidine and were sacrificed after 6 months. While the determination of the fraction of labelled cells immediately after the injection of tritiated thymidine gave an indication of the degree of cell formation (cell formation test), the fraction of the labelled cells that still persisted even after 6 months was used as an index of cell retention (cell retention test). The different tissues were then classified into three groups, viz. stable cell populations, expanding cell populations and renewing cell populations, depending on whether they contained labelled cells in either, both or neither of these tests.

Stable/

Stable Cell Populations:-

In this group of tissues, although no labelled cells were found in the cell formation test in the adult animal, labelled cells were seen in 3-day old animals injected with tritiated thymidine and sacrificed after 6 months. These were cell populations that showed proliferative activity at birth which ceased after a short while. Striped, smooth and cardiac muscle, and nervous tissue were included in this group.

Expanding Cell Population:-

This group showed a small number of labelled cells in both cell formation and cell retention tests indicating a slow increase in the number of cells in the population. However, the rate of this increase appeared to diminish with increasing age. Tissues included in this category were proximal convoluted tubules of kidney, paranchymal cells of liver, epithelial cells of thyroid, acinar and islet cells of pancreas and adrenal cortical and medullary cells.

Renewing Cell Populations:-

These populations showed numerous labelled cells in the cell formation test in the 3-day old as well as adult animals, but failed to show any labelled cells in the cell retention test. The labelled cells that were observed initially were considered to have been lost in the course of time as a result of the constant renewal of the elements in these cell populations. Tissues included in this group were epithelium of tongue, skin, intestine, cortical cells of thymus and the connective tissue of skin.

Although it is apparent that the classification of Leblond et al. (1959) did not include every tissue in the body, it formed a simple classification of the various cell populations depending on their kinetic behaviour. In fact Leblond and co-workers have made further studies to include a number of other/

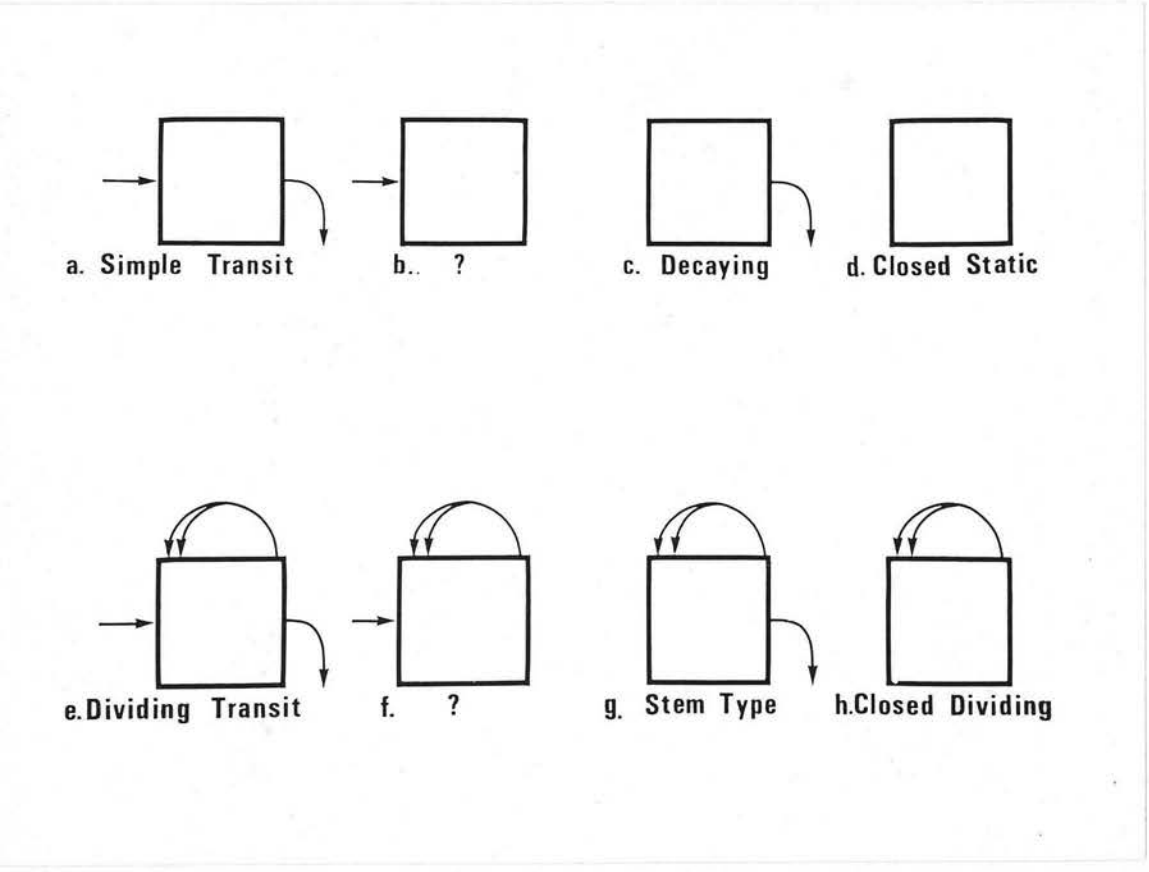

Fig. I.

From Gilbert and Lajtha (1965).

other tissues and also to re-allocate certain cell populations (Messier & Leblond, 1960; Leblond, 1964).

It is interesting to note that the scheme of classification adopted by Leblond et al. (1959) was not unlike that proposed by Bizzozero (1894). While the labile and perennial tissues of Bizzozero correspond to the renewing and stable cell populations of Leblond et al., the cell populations recognised by the latter workers as expanding were in fact included in the stable tissues of Bizzozero.

It was not surprising that Bizzozero considered these populations as stable, for it is extremely difficult to recognise the low rate of proliferative activity in these tissues by mere observations on the presence or absence of mitotic figures. Incorporation of tritiated thymidine, however, is a more sensitive technique for the recognition of proliferative activity, as the proportion of cells in DNA synthesis at any one time is much higher than that in mitosis.

Gilbert & Lajtha (1965), taking into consideration the possibilities of cell division within a cell population and the cell gain from, or cell loss to, other populations, put forward a scheme of the various combinations of these possibilities, representing hypothetical as well as those populations that are present in the body. The scheme of Gilbert & Lajtha (1965) is illustrated in figure 1. The arrow entering the box indicates cells entering the population and the arrow leaving indicates cells leaving. Double-ended arrows leaving the box and re-entering indicate cell division within a population.

A simple transit population (fig. 1a) is one in which there is no proliferation taking place, with the cells merely passing through it. Examples of these are reticulocytes, functional cells of the intestinal villi, mature cells of the squamous stratified epithelia, etc. At the present moment no cell population is known, in actual practice, showing the combinations indicated in fig. 1 b & f and hence they are considered/

considered hypothetical. An example of a decaying cell population (fig. 1c) is the ovary from which cells are being released without any cell division after birth. Neurons of the central nervous system which do not undergo cell division in the adult are considered to be of the closed static type (fig. 1d). The dividing and maturing cells in the bone marrow are examples of the dividing transit type (fig. 1e). A stem cell population (fig. 1g) is one that is self-maintaining, i.e. they divide to maintain the number of cells in the population without any cell gain from other sources. In addition to maintaining their own numbers they also yield cells which migrate out of the population and differentiate. Examples of this type are stem cells of the bone marrow, cells of the intestinal crypts, stem cells of squamous stratified epithelia. Cells in tissue culture, regenerating liver cells and tumours from which there is no cell death or metastasis were considered examples of the closed dividing type of cell population (fig. 1h).

Steady State Renewal Systems

The cell populations in the adult that are constantly being renewed, and in which the average rate of cell production is in balance with the rate of cell loss, so that the size of the population remains fairly constant are referred to as steady state renewal systems. Typical examples of such systems are the epithelia of the skin, oral mucosa and gastro-intestinal tract, and the haemopoetic system.

The mature cells of a renewal system have a definite average life span, after which they are removed from the cell population and are replaced by younger cells arising from the proliferative activity of the stem cells, in order to maintain constancy of cell numbers. This constancy of cell numbers should not be regarded as absolute in the mathematical sense. During the life span of the animal itself, there is an increase in cell number during the post-natal period until adult stage and a decrease during ageing. However, this increase or decrease is negligible in the adult state for relatively long periods of time, so that the assumption of steady state is justifiable. It need not be emphasised that the real systems seen in practice are almost always found to be departures from an ideal model.

For purposes of the study of cell population kinetics, it is often convenient to divide the steady state renewal system into a number of compartments. The simplest example would consist of two compartments, viz. a stem cell compartment and a mature cell compartment. However, in practice, the transition between stem cells and mature cells is gradual and hence there is often a compartment of maturation interposed between the two. Such a three compartment steady state renewal system is diagramatically shown in figure 2.

The/

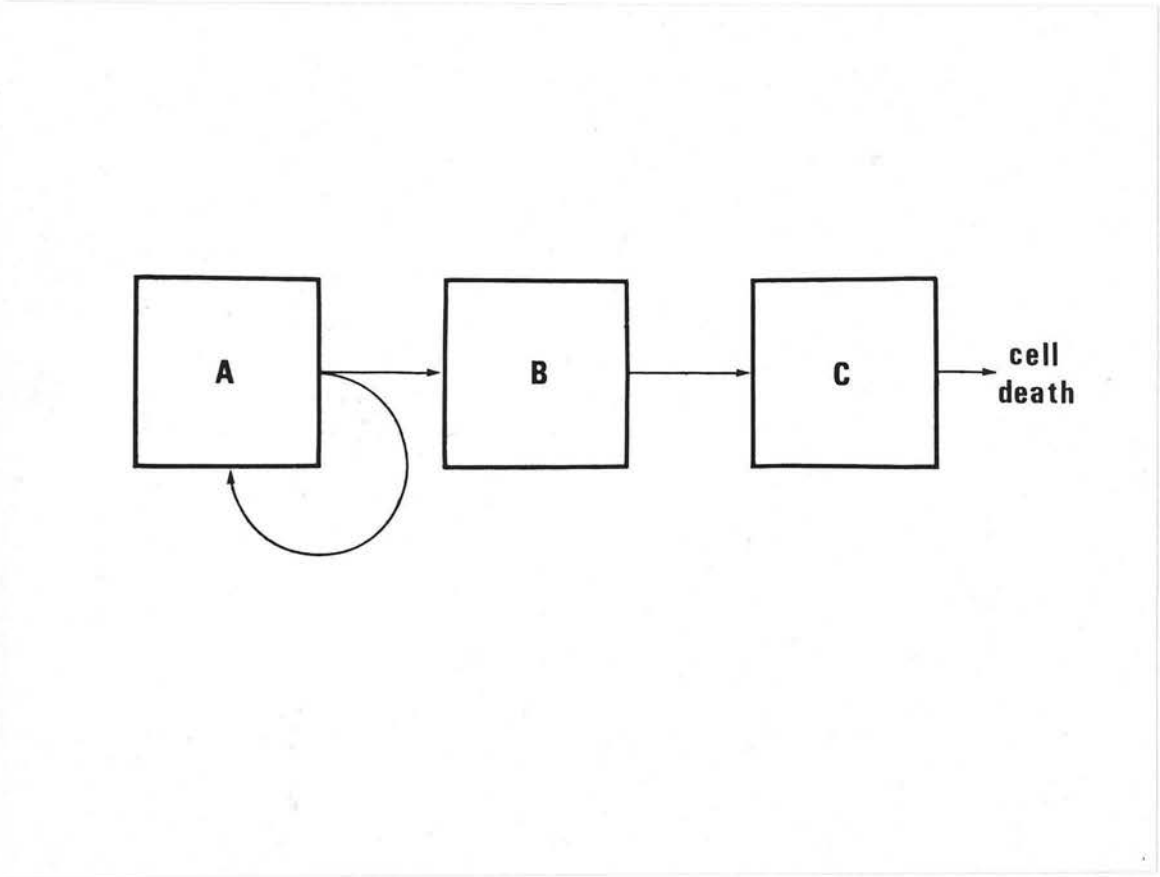

Fig. 2.

The boxes marked A. B and C represent the three compartments in the system, A being the stem cell compartment, and B & C the maturing, and mature cell compartments respectively. In actual practice, however, it is not always possible to demarcate the boundary between one compartment and another. While the stem cell compartment could be clearly demarcated spatially in the epithelium of the intestine, oesophagus, etc., being confined to the intestinal crypts in the former and the basal cell layer of the latter; the various compartments in the bone marrow, for example, are thoroughly mixed with each other so that there is no boundary as such in the spatial sense, and the cells of the various compartments could only be recognised by their morphological characteristics. The "migration" of cells from one compartment to another in this case would then be a mere change in the morphology of the cells. In systems which have clearly demarcated compartments such a migration would involve physical movement of the cells.

The proliferative activity is confined to compartment A (fig. 2) and these cells steadily progress through the cell cycle leading to cell division. The events of the cell cycle will be discussed in the next section. Cells move out from compartment A to compartment B at the same rate as new cells are being generated in the former compartment. Thus the cell gain in compartment A over a period of time is zero. Also the efflux of cells from compartment B is the same as the influx into this compartment, so that the total number of cells in B remains fairly constant. Again the rate of cell loss from compartment C is equal to the rate at which cells enter compartment C. In other words, not only the size of the population but also the size of each of the compartments remains fairly constant.

The time that elapses between the cell entering a compartment and the cell leaving the compartment is referred to as the transit time. The individual cells of a system do not have exactly the same transit time, but possess a distribution of transit times, and the average time spent in each/

each compartment is referred to as the mean transit time (Quastler, 1963). In a non-dividing compartment like compartment B or C (fig. 2) the transit time will also be equal to the turnover time. Turnover time is defined as the time taken for the replacement of the number of cells equal to the size of a compartment (Leblond & Walker, 1956). The turnover time for a stem cell compartment is commonly referred to as the generation time.

With increasing complexity of the renewal systems, there will be an increase in the number of compartments, and cell division may take place in one or more cell compartments in addition to the stem cell compartment. In such maturing and dividing cell compartments the rate of efflux would be equal to influx plus the rate of generation of cells in the compartment in order to maintain constancy of cell numbers and hence the steady state.

Cell renewal systems have been discussed by Leblond & Walker (1956) and Bertalanffy & Lau (1962).

The Cell Cycle

The cell cycle is defined as the interval between completion of mitosis in a cell and completion of a subsequent mitosis in one or both of the daughter cells. Howard & Pelc (1953) were the first to describe the various phases of the cell cycle. They divided the cell cycle into four distinct phases, viz. the G1 phase, which is the interval between completion of mitosis and onset of DNA synthesis, the S-phase, during which duplication of DNA takes place, the G2-phase, which is the interval between cessation of DNA synthesis and onset of mitosis, and finally mitosis, which included the four stages from prophase to telophase. Before the original work of Howard & Pelc (1953), the life cycle of cells was merely divided into a long period referred to as the interphase and a short period of mitosis.

Although/

Although a few modifications have been suggested to the description of the cell cycle by Howard & Pelc (1953), the four-phase cycle as described by them is widely accepted in its original form.

Bullough (1963, 1965) proposed that the interphase should be divided into three main phases, viz. the apophase, which is the period immediately following mitosis, the dichosphase, which was described by Bullough as the period during which the cells make the decision to progress towards division, and the prosphase, which was described as the period from the end of dichosphase to the onset of mitosis. The prosphase was further sub-divided by Bullough into an early prosphase, the DNA synthetic phase and an antephase.

The apophase, dichosphase and the early prosphase of Bullough (1963, 1965) together constitute the G1 phase of Howard & Pelc (1953), and the post-synthetic gap denoted by Bullough as the antephase is merely the G2 phase of Howard & Pelc.

The earliest recognisable event, using present day techniques, in the movement of cells participating in the cell cycle towards mitosis is the onset of DNA synthesis. Although considerable work is being done, very little is known regarding the sequence of biochemical events that take place prior to onset of DNA synthesis which lead to the progression of the cells towards mitosis (Baserga, 1968). Hence the sub-divisions of the cell cycle as proposed by Bullough does not seem necessary at the present moment, except perhaps on a speculative basis.

In certain cell populations few cells have been known to stay quiescent in the G1 phase, G2 phase or in both (Gelfant, 1962 & 1963; Starkey, 1963; Cameron & Cleffmann, 1964). Such cells were considered to be in a Go phase. Although some workers consider that these are cells with an extended duration either in the G1 or G2 phases or both, there seems to be some experimental evidence to suggest that they are a distinct entity of cells which, on appropriate stimulation, progress towards mitosis (Baserga, 1965)..

Even/

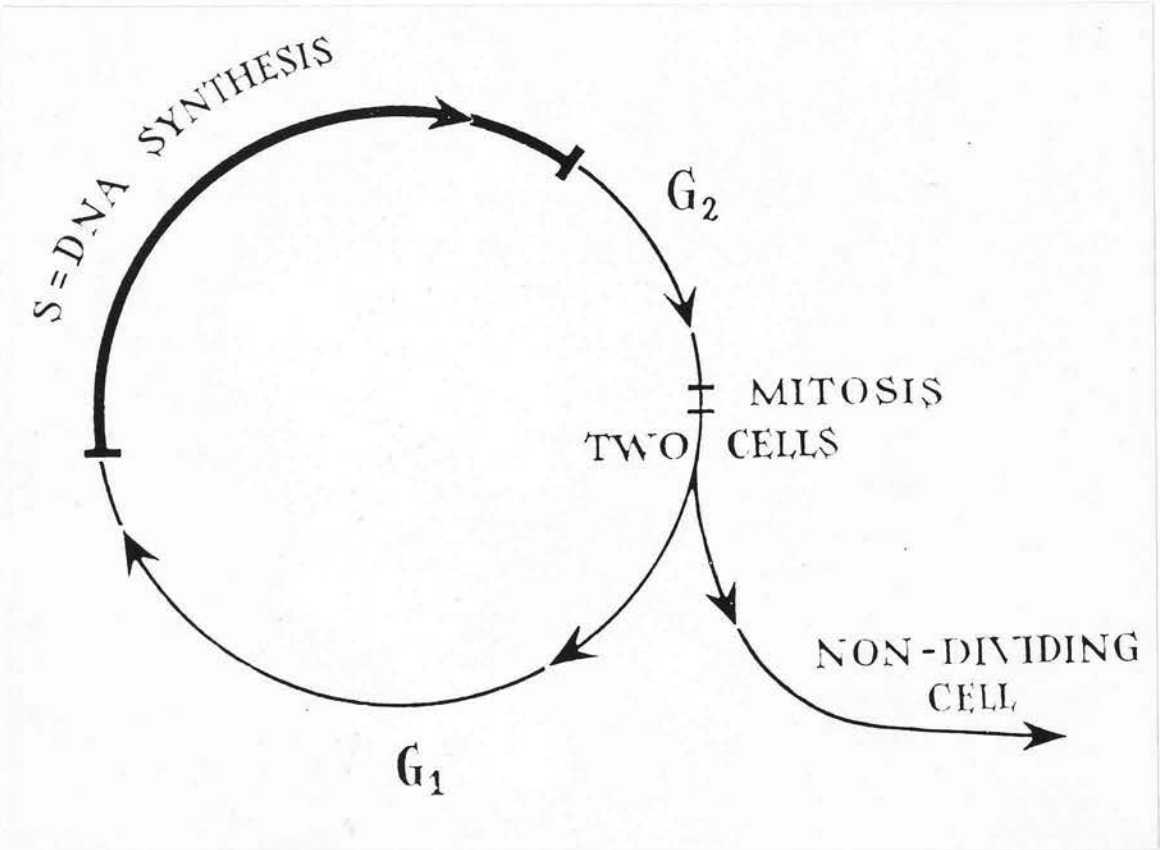

Fig. 3.

From Baserga (1965).

Even if it is possible to demonstrate beyond doubt that the Go cells described in certain populations are a distinct entity, it does not seem necessary to include such a phase in the general picture of the cell cycle, and hence the original description of the cell cycle by Howard & Pelc is adhered to in this work, and the phases of the cell cycle are illustrated in figure 3.

G-1 Phase:-

The G1 phase variously referred to in the literature as the first growth phase, the first gap, or the post-mitotic pre-synthetic gap, is the interval between the end of telophase to the onset of DNA synthesis in cells that continue to participate in the cell cycle following mitosis. The G1 phase is also the phase of most variable duration. While the Xenopus eggs (Graham, 1966), slime mold (Nygaard et al., 1960), and a few mammalian cells (Baserga, 1963; Starkey, 1963; Robins & Scharff, 1967) do not have distinguishable G1 phase, the G1 phase may last for a few hours or days in other cell populations (Quastler & Sherman, 1959; Sherman et al. 1961; Dhawan & Toto, 1965).

S-Phase:-

The S-phase, or the DNA synthetic phase, is the interval during which the cells duplicate the DNA content of their nuclei in preparation to division. The cells in S-phase are extremely useful "markers" in the study of cell population kinetics for they could be labelled by the administration of a radioactive DNA precursor and their fate followed by autoradiography. Although the duration of the S-phase was originally thought to be fairly constant and very close to 8 hours in mammalian cells (Mendelsohn et al., 1960; Painter & Drew, 1959; Young, 1962; Cameron, 1964), the number of exceptions/

exceptions have been increasing over the past few years so that the original assumption is no longer tenable. While most mammalian cells have an S-phase of 5-12 hours duration, the S-phase in others is known to be 20 hours and more (Sherman et al., 1961; Bresciani, 1964).

G2-Phase:-

The G2 phase is the interval between the completion of DNA synthesis and the beginning of mitosis. In most mammalian cells the length of the G2 phase is comparatively short and has less variability than the G1 and S phases. However, some cell populations are known to have quiescent G2 cells (Gelfant, 1962; Starkey, 1963; Cameron & Cleffmann, 1964). Although protein and RNA synthesis takes place in the cells there is no synthesis of DNA during the G2 phase.

Mitosis:-

Mitosis is the only visually distinguishable phase in the cell cycle. It is customarily sub-divided into the four stages, viz. prophase, metaphase, anaphase and telophase. Protein and RNA synthesis almost come to a standstill in mitosis except during early prophase and late telophase (Feinendegen et al., 1960; Das, 1963). Out of the two daughter cells that result at the end of each mitosis, one or both or none of them may continue to participate in the cell cycle (Marques-Pereira & Leblond, 1965).

Although the factors that decide the fate of the individual daughter cells and the maintenance of steady state are not clearly understood in many cell populations, in the squamous stratified epithelia at least the cells seem to leave the proliferative compartment at random being pushed out by the pressure exerted by the crowding produced by generation of new cells, so that for every new cell that is formed a cell moves out in order to maintain the steady state (Leblond et al. 1964).

The/

The Control of Mitotic Activity:-

Contrary to the older idea that mitotic activity was dependant on the stimulation of tissues by some humoral factor, the consensus of opinion today is that all cells are capable of division, and that the regulation of cell division is due to the presence of specific mitotic inhibitors within each type of tissue (Bullough, 1962, 1965). The presence of tissue specific mitotic inhibitors was first demonstrated by Saetren (1956 and 1963) in liver and kidney, and the existence of such inhibitors in skin was demonstrated by Bullough & Lawrence (1961) and Finegold (1965). The mitotic inhibitor in mouse skin was subsequently extracted by Bullough & Lawrence (1964) and the term chalone has been proposed for these tissue specific mitotic inhibitors. Chalones were subsequently extracted from skin of hairless mice (Iversen et al., 1965) and from granulocytes (Rytoma & Kiviniemi, 1964). In a series of studies (reviewed by Bullough, 1965) Bullough and co-workers have shown that the amount of mitotic inhibition achieved depends on the concentration of chalone in a particular tissue, and that the active mitotic inhibitor was an unstable complex formed by chalone and adrenaline. Chalone concentration of a tissue depends on the balance between the rate of production of chalone within the tissue, and the rate of diffusion or removal from the tissue.

The chalone is thought to be produced by the mature cells, and the control of mitotic activity of the progenitor cells through feedback stimuli from mature cells had been proposed earlier (Iversen, 1961; Mercer, 1962).

On the chalone hypothesis, the increased mitotic activity after wounding is explained as due to a lowering of the local concentration of chalone as a result of the damaged cells being unable to synthesise chalone. This lowering of chalone concentration partially removes the restraint on mitotic activity and hence more cells undergo cell division. Bullough (1962) stated that greater degrees of injury result in much lesser concentrations of chalone and hence a greater stimulation of mitotic activity.

There/

There has been an increasing amount of support for the hypothesis of chalone control of mitotic activity (Hansen, 1967a; Iversen & Elgjo, 1967).

Factors Influencing Mitotic Activity.

Age:-

While a number of studies on the effect of age on the mitotic activity of squamous stratified epithelia have been made, the results obtained appears diverse. While Katzberg (1952); Whitley & Horton (1963) and Fisher (1967), observed a steady decrease in the mitotic activity of the skin with increasing age, Thuringer & Cooper (1950) and Bertanffy et al. (1965) reported an increase with age. Meyer et al. (1956) observed a higher mitotic index in the epithelium of human gingiva in older individuals than in the younger age group.

Sharav & Massler (1967),studying the age changes in the epithelium of the palate and tongue in rats through the determination of the labelling indices, found that while there was a decrease in the number of progenitor cells under unit length of the epithelium in old age, there was an accompanied increase in the percentage of labelled cells. Although they recognised the possibility that the increase in the percentage of labelled cells could be due to an increase in the duration of DNA synthesis, they implied that the increase in the percentage of labelled cells that accompanied a decrease in the number of progenitor cells per unit length of epithelium was an attempt to maintain a constant rate of turnover.

Hormones:-

Reviewing the literature on the effect of hormones on mitotic activity, Bullough (1962) stated that only two groups of hormones were of importance in relation to mitotic activity. One was the glucocorticoids, adrenaline and noradrenaline which have an anti-mitotic action on a number of tissues, and the other oestrogens and androgens which have a powerful mitogenic effect/

effect on the cells of certain "target tissues" and a slight effect on few other tissues. The variations in the mitotic activity during the oestrous cycle in females is a result of the fluctuation in the levels of sex hormones.

Bullough (1962) also stated that the importance of other hormones like thyroxin, insulin and pituitary hormones in relation to mitotic activity was only so far as they ensure the balanced working of the metabolic complex of the body.

Nutrition:-

A number of studies have shown that restricted intake of food leads to a decrease in the mitotic activity of a number of tissues (Rabinovitch, 1928; Loeb, et al., 1939 Bullough & Ebling, 1952).

Bullough & Ebling (1952) stated that even when the mitotic activity of mouse epidermis was about 25% of the normal during starvation, the thickness remained constant. This indicates the preciseness of the regulation of steady state by a decrease in the rate of cell loss, and an increase in the mean life span of the mature cells.

Bullough (1962) considered that the depression of mitotic activity in starvation was probably due to the anti-mitotic action of adrenaline which is secreted as a result of the stress experienced by the starving animal.

Diurnal Variation:-

Variation in mitotic activity over the 24-hour period has been observed in a number of tissues. While this diurnal variation is most prominent in moderately proliferating tissues, it is almost undetectable in tissues with very high or very low mitotic activity (reviewed by Bullough, 1965). Bullough & Lawrence (1966) and Hansen (1967b) have demonstrated that not only the mitotic rate but also the mitotic duration shows a 24-hour periodicity. They also showed that while adrenalectomy produced an increase in mitotic rate and an abolition of the diurnal variation, injection of adrenaline into sleeping mice reduced mitotic rate.

The/

The current explanation for the existence of a diurnal rhythm is given in terms of the variation in the chalone adrenaline-complex during sleep and wakefulness (Bullough, 1965). When the animal goes to sleep there is a fall in the amount of adrenaline circulating and the concentration of chalone-adrenaline-complex in a particular tissue is slightly decreased and consequently there is an increased mitotic activity. During wakefulness just the opposite happens. Diurnal variations in the secretion of adrenaline have also been described (Karki, 1956), and the diurnal rhythm in mitotic activity is, in fact, the inverse of this rhythm.

Methods for the Study of Cell Proliferation

Mitosis being the only morphologically recognisable phase of the cell cycle, classical studies on cell proliferation have been based on the recognition of mitotic figures in the tissues and the determination of their number per "unit" of the tissue (Bizzozero, 1894; Thuringer, 1928; Cowdry, et al. 1944). However, with the advent of radioactive isotopes and the refinement of the techniques of autoradiography, it has been possible to study cell proliferation at yet another phase of the cell cycle, viz. the S-phase.

Studies of Cell Proliferation Based on Mitotic Activity:-

The mitotic activity of the tissues have been expressed in various forms. Spain (1915) and Thuringer (1924) expressed the number of mitotic figures in the epidermis per unit area of the epithelium in histological sections, while Thuringer (1928) and Cowdry et al. (1944) expressed mitotic activity as the ratio of the number of mitosis to the total number of cells. Kiljunen (1956), Marwah et al. (1960), Meyer et al. (1960), Gargiulo et al. (1961), Bertalanffy et al. (1965), Soni et al. (1965) and Fisher (1967), have defined mitotic index as the total/

total number of dividing cells per 1000 nucleated or viable cells. Beagrie (1966), Blenkinsopp (1967) and Brown & Berry (1968), studying the mitotic activity of squamous stratified epithelia, have expressed the number of mitoses as a percentage of the total number of basal cells counted. Muhlemann et al. (1959), Renstrup (1963) and Hansen (1967b) defined mitotic index as the number of mitoses per unit length of the basement membrane, while Main (1969), expressing the mitotic activity of cyst epithelia by the same method, called it the mitotic value. While Evensen (1962), Iversen (1966) and Bullough & Lawrence (1966) expressed the mitotic activity of the epidermis as a function of the surface length of the epithelium, Loe & Karring (1969) have suggested that mitotic index should be defined as the number of dividing cells per square mm. of the surface of the epithelium.

Such diversity in the expression of mitotic activity in squamous stratified epithelia has made it difficult to interpret and compare the results of various workers.

While measurements of mitotic activity based on length or area of the epithelium may be satisfactory for relative comparisons of mitotic activity between various tissues or alterations of mitotic activity within a tissue, the study of cell renewal in tissues should, however, involve cell numbers, and in these cases it becomes necessary to express mitotic activity as a function of the number of cells.

The mitotic index in whatever way it is expressed is a measure of the number of cells in mitosis at any one time. The rate of mitosis which is defined as the number of cells entering or completing division per unit time would be a more appropriate measure of the proliferative activity of a tissue (Evensen, 1962). The mitotic rate depends on the mitotic index as well as the mitotic duration which is defined as the time taken for completion of each division. Thus mitotic rate = mitotic index/mitotic duration, and comparisons of mitotic activity of tissues through mitotic indices become accurate only when the respective mitotic durations are similar.

While/

While determination of mitotic duration by direct observation is not feasible in the vivo situation, the advent of cytostatic agents which arrest mitosis at the metaphase stage have made it possible to determine the rate as well as the duration of mitosis, by accumulation of metaphase-figures over a period of time. Colchicine and its derivative colcemid have been the most popularly used agents (Eigsti & Dustin, 1955; Leblond, 1959; Hooper, 1961). Leblond (1959) listed that the following requirements should be satisfied before a cytostatic drug could be made use of in the study of cell proliferation:

1) the drug should not act at stages prior to metaphase,
2) the time at which the drug takes effect should be known for the route of administration,
3) the effect of the drug should be longer than the duration of the experiment,
4) the duration of the experiment should be shorter than the generation time of the cell population.

Colcemid is much less toxic than colchicine (Evensen, 1962; Hell & Cox, 1963) and has been widely used in studies on cell proliferation.

Unfortunately, the syrian hamster is resistant to the action of colchicine or colcemid (Orsini & Pansky, 1952). Midgley et al. (1959), studying the nature of this resistance, injected colchicine into hamsters bearing transplanted heterologous tumours. While the heterologous transplanted tissues showed metaphase-arrest of mitosis, the hamster tissues did not respond to the action of colchicine. Embryonic hamster cells in tissue culture and anaplastic carcinoma of hamster cheek pouch in vivo also showed similar resistance to the action of colchicine. From these studies Midgley et al. (1959) concluded that the resistance of the hamster tissues to the action of colchicine was due to an inherent/

inherent resistance possessed by the cells rather than due to systemic inactivation of the drug. The explanation for this resistance is that the plant Colchicum autumnale from which colchicine is extracted, has a wide distribution throughout Syria, the country of origin of the Syrian hamsters. These animals, by frequently nibbling at the corms of this plant, have acquired the specific resistance during the course of evolution (Bertalanffy, 1967).

In the search of an alternative to Colchicine in the studies on cell proliferation in the hamster, it was thought that the alkaloid vinblastine (Vincaleukoblastine) (extracted from Vinea Rosea linn), which possessed properties of producing metaphase arrest in vitro (Palmer et al. 1960) as well as in vivo (Cutts, 1961; Cardinalli et al., 1961) might prove suitable. The thoughts on the possibility of making use of Vinblastine as a cytostatic agent were further reinforced by the suggestion of Bertalanffy (1967) that this drug may be of use in the study of cytodynamics in the hamster if the effective dosage etc. could be standardised. Studies on the cystostatic action of this drug on the oral epithelia of hamsters will be described later.

While mitotic accumulation produced by cytostatic drugs provides a convenient way of measuring rate of mitosis, its duration, and renewal times in various tissues, this method has its own limitations. One important problem is that, with increase in time after the injection of the drug, the arrested metaphases tend to disintegrate leading to a slight departure from the linearity of the increase in the arrested metaphases over time (Hooper, 1961). Also it is not possible to study the migration, and hence the fate of the dividing cells using this technique.

The Study of Cell Proliferation Using Tritiated Thymidine:-

The first labelled thymidine to become available was 15_N thymidine and was used by Reichard & Eastborn (1951) who demonstrated that thymidine was specifically incorporated into

DNA/

DNA. They also predicted that labelled thymidine would be of value in experiments intending to label DNA alone. The findings of Reichard & Eastborn were confirmed by Freidkin et al. (1956) and Amano et al. (1959) using 14_C and 3_H labelled thymidine.

The first successful synthesis of tritiated thymidine was reported by Taylor et al. (1957) and Firket & Verley (1958). Thereafter tritiated thymidine has been used in innumerable studies on cell proliferation, as well as in studies of cellular activity at a sub-cellular level.

Tritium is a heavy isotope of hydrogen with an atomic mass of three. The combination of two neutrons and one proton in the nucleus of the tritium atom being unstable, it undergoes disintegration to yield an atom of helium containing two protons and one neutron with escape of an electron (beta-particle).

The beta-particles emitted by tritium have a maximum range of 8 microns (average 1.5 microns) and a maximum energy of 18.5 keV. The half-life, i.e. the time taken for the radioactivity to decay to half its initial value, of tritium is 12.26 years (cf. half-life for 14_C is 5600 years) (Lajtha & Oliver, 1959). While the short range of the beta-particles emitted by tritium gives excellent autoradiographic resolution even at sub-cellular levels (Taylor et al., 1957), slices of labelled cells lying at the deep end of routine tissue sections may fail to be registered in the emulsion for the same reason (Evensen, 1962).

Tritiated thymidine is commercially available in a range of specific activities and with a high radiochemical purity ($>$ 98%).

Tritiated thymidine is a relatively stable compound and can withstand heating up to 120°C. without significant breakdown of the molecule or loss of label (Crowter et al., 1960). However, when stored for a long period of time at room temperature there is a very slow hydrolysis (1-2% per year) taking place at the N-glycosidic bond (Evans & Stanford, 1963). Contamination of the solutions with micro-organisms may result in the loss of label by enzymatic breakdown of the thymidine molecule.

There is also a constant self-decomposition of the tritiated thymidine molecule as a result of radiation effects. Bayly & Weigel (1960) recognised four radiation effects that may contribute/

contribute towards self-decomposition:-

1) Primary (internal) radiation effect produced by the natural decay of the isotope.

2) Primary (external) radiation effect due to the direct interaction of the emitted beta-particles with the molecules.

3) Secondary radiation effect due to the interaction of ionised product with the molecules.

4) Chemical effects.

The overall rate of self-decomposition varies from about 0.5 to 2% per month and depends on the specific activity of the solution and the position of $^{3}_{H}$ atom in the pyrimidine ring (Evans & Stanford, 1963; Evans, 1966). The rate of decomposition is increased by an increase in temperature and it is usually satisfactory to store tritiated thymidine at 0°C. or 4°C. Lower temperatures do not reduce the rate of decomposition very much unless, of course, it is possible to store it at -196°C., at which temperature the rate is very much reduced (Evans, 1966). It is, however, not advisable to store tritiated thymidine for long periods of time, especially in high specific activities. Wand et al. (1967) have stressed the possible effects of the decomposition products of tritiated thymidine on its use as a tracer for DNA synthetic activity.

Problems associated with the stability of radioisotope labelled compounds, and their storage have been discussed in detail by Bayly & Evans (1968).

Tritiated Thymidine as a Tracer in Studies of Cell Proliferation

Cronkite et al. (1959 a & b) listed a number of conditions that have to be satisfied, or assumptions to be made in the use of tritiated thymidine as a tracer in studies of cell proliferation. They are listed below although not necessarily in/

in the same order as that of Cronkite et al.:-

1) tritiated thymidine is uniformly distributed throughout the body after administration, and is promptly incorporated into DNA,

2) the time for which tritiated thymidine is available is short in comparison to the duration of DNA synthesis,

3) there is no significant injury to the cells on account of the emitted beta-particles,

4) synthesis of DNA in cells normally destines them to divide,

5) DNA turnover is solely due to mitosis and death,

6) tritium label does not exchange under biological conditions,

7) thymine base does not exchange after incorporation into DNA,

8) reutilization of tritium labelled breakdown products from DNA of dead cells is insignificant.

Although a number of these assumptions have been debated, there is not sufficient conclusive evidence to raise serious objections to the use of tritiated thymidine as a tracer, and in most cases it fulfils the aforementioned conditions.

Availability of Tritiated Thymidine After a Single Injection:-

There have been a number of studies on the availability of tritiated thymidine for incorporation into DNA after administration by the various routes.

Intra-venous route: Rubini et al. (1960), studying the fate of intra-venously administrated tritiated thymidine in humans, found that about 90% of the injected thymidine was either incorporated into DNA or broken down during the first two minutes following the injection. Grain count analysis of autoradiographs prepared from bone marrow cells showed that maximal incorporation of label was reached one hour after the injection although the increase in the intensity of the labelling from 10 to 60 minutes after the injection was rather small.

Staroscik/

Staroscik et al. (1964) reported that there was significant labelling of mouse mammary tumours 40 minutes after intra-venous injection of tritiated thymidine even though it was only about 1 to 2% of the total uptake. They also stated that about 95% of the uptake after a single intravenous injection took place during the first 25 minutes.

Chang & Looney (1965), studying the incorporation of tritiated thymidine into regenerating liver cells, found that there was a rapid disappearance of labelled thymidine from blood during the first 10 minutes following injection, and it was accompanied by increase in the radioactivity of the acid soluble fraction, and the DNA of the liver cells. They also stated that the maximum rate of incorporation of tritiated thymidine into DNA occurred during the first 30 minutes.

Skougaard (1965), reporting on the availability of tritiated thymidine after intra-venous, intra-peritoneal and intra-muscular injections in marmosets, stated that a greater amount of thymidine was available for incorporation into DNA following administration by the intra-venous route than by the other two routes. However, the reproduceability of the autoradiographic grain counts was poor owing to the rapid incorporation of thymidine following intra-venous injection. Skougaard stated that total clearance of tritiated thymidine from plasma occurred about one hour after intra-venous injection.

Intra-peritoneal injection: Quastler & Sherman (1959), studying the labelling of the intestinal crypt cells of mice at varying intervals after intra-peritoneal injection of tritiated thymidine by autoradiography, found that labelling was recognisable as early as 5 minutes after injection, and that the maximum intensity of labelling was reached between 10 to 20 minutes after injection.

Skougaard/

Skougaard (1965), reporting that the clearance of tritiated thymidine from plasma occurred about 60 minutes after intra-peritoneal injection in marmosets, stated that the maximal labelling as assessed by autoradiographic grain counts was achieved at about the same time. However, in small animals (mice) the total clearance of tritiated thymidine from plasma is complete within 30 minutes following an intra-peritoneal injection (Skougaard & Stewart, 1967).

Intra-muscular route:

Skougaard (1965) reported that the amount of tritiated thymidine available after intra-muscular injection was greater than that following an intra-peritoneal injection. He suggested that this was probably due to the fact that a major part of the tritiated thymidine administered intra-peritoneally being absorbed by the portal veins, has to pass through the liver, which is the main site of thymidine catabolism, and hence part of it may be broken down. He also stated that the total clearance of thymidine from plasma occurred only 2 hours after the injection.

While this comparatively longer plasma clearance time made this route unsuitable for purposes of pulse labelling, Skougaard stated that the intra-muscular route gave the best results regarding grain count reproduceability.

Sub-cutaneous route:

Messier & Leblond (1960) reported that after sub-cutaneous injection in the rat, the tritiated thymidine activity in the plasma reached its peak in 20 minutes. This was followed by a rapid fall of activity leading to total plasma clearance by 60 minutes after injection.

The investigations outlined above have shown that tritiated thymidine is available to the cells only for a relatively short time after administration by the intra-venous, intra-peritoneal, or sub-cutaneous routes and hence satisfies the conditions of a pulse label.

The/

The Mitogenic Effects of Thymidine:

Greulich et al. (1961) reported that administration of exogenous thymidine produced a significant increase in the number of mitotic figures observed in the duodenal epithelium of mice ½ to 6 hours after the injection. They concluded that this was due to the shortening of the duration of the S-phase which enabled the cells to move into mitosis at a faster rate.

Baserga & Kisieleski (1962a), repeating the experiments of Greulich et al. using even higher doses of tritiated thymidine, were unable to find any stimulation of mitosis in the crypt cells of the small intestine of mice or in Ehrlich ascites tumour cells growing in the peritoneal cavity of mice. They estimated that the total thymidine pool of a 30 gram mouse was about 50 micro-grams and suggested that the quantities of exogenous thymidine injected in tracer studies were too small to bring about undue fluctuations of the endogenous thymidine pool. Similar conclusions were also made by Messier & Leblond (1960).

Beagrie (1966) using the epithelium of the tongue and palate of mice as the test tissues, reported a significant increase in the mitotic indices in animals sacrificed 3 hours after the injection of labelled as well as unlabelled thymidine.

Blenkinsopp (1967), studying the rate of entry of cells into mitosis in the epithelium of the tongue and oesophagus in mice, over a period of 8 hours following injection of tritiated thymidine, reported that there was no significant differences in the mitotic rates of the control and experimental animals. He stated that the absence of mitotic stimulation in his experiments was due to the minute amounts of thymidine used (0.074 micro-gram per gram body weight). He was of the opinion that the amounts of thymidine administered when tritiated thymidine is used as a label should not exceed 0.1 micro-gram per gram body weight.

The/

The observations of Beagrie (1966), that the degree of mitotic stimulation in the epithelium of the tongue and palate of mice receiving 10 micro-grams of thymidine was greater than those receiving only 7.3 micro-grams, would lend support to the importance of the amount of thymidine administered in the consideration of a possible mitogenic effect of this substance. In the above study, however, mitotic stimulation was observed in both experimental groups, probably as a result of the large amounts of thymidine utilized.

The discrepancy between the results of Greulich et al. (1961) and those of Baserga & Kisieleski (1962a), however, remains unexplained.

In the light of the above discussion it seems reasonable to assume that the amounts of exogenous thymidine introduced into the hamsters in the present study (0.048micro-grams per gram body weight) would not influence cell proliferation.

Radiobiological Effects of Tritiated Thymidine:

While there is considerable reduction in the survival time of cells in tissue culture as a result of radiation effects when exposed to tritiated thymidine (Drew & Painter, 1962; Whitmore & Gulyas, 1966), there does not seem to be any hazardous effects in animal experiments where doses of 1-2 micro-curie/gram body weight are used (Mendelsohn, 1960). This is probably a result of the fact that a large portion of the injected material is degraded and excreted, with only a small proportion being incorporated into the cells. Skougaard (1965) stated that the radioactive DNA content of the skin and gut of marmosets accounted for only 0.1% and 0.5% of the injected activity respectively. Assuming that at least 50% of the mitotic activity is present in the epithelium of the skin and gut (Messier & Leblond, 1960), indeed a small amount of the injected thymidine is taken up by the cells.

The/

The administration of much larger doses of tritiated thymidine, however, produced radiation damage and arrest of growth (Johnson & Cronkite, 1959; Kisieleski et al., 1964). Animals injected with tritiated thymidine and observed over a long period of time tend to develop a variety of malignant tumours, and there seems to be a higher incidence of tumours in animals receiving larger doses (10 micro-curie) than those receiving 1 micro-curie per gram body weight (Baserga et al., 1962).

However, the radiation effects are negligible in short term animal experiments using 1-2 micro-curies per gram body weight, so that it becomes reasonable to assume that cell proliferation is not upset by the introduction of the radioactive DNA label.

Reutilization of Tritiated Thymidine:

Steel & Lamerton (1965), studying the turnover of tritiated thymidine in tissues of the rat, proposed that the reutilization of labelled DNA could take place by four main methods:-

1) incorporation of DNA breakdown products from dead cells of the same tissue, i.e. local reutilization,

2) systemic reutilization of breakdown products of DNA from one or more tissues,

3) the special case of the gastro-intestinal tract in which the DNA of cells which are sloughed off into the lumen may be reabsorbed lower down the tract and preferentially incorporated into the epithelial cells. If the absorbed material is reutilized systemically, it becomes equivalent to case 2,

4) the special case of the bone marrow, in which during erythropoiesis the nucleus is "discarded" at the orthochromatic stage and a local reutilization of DNA is highly probable.

Baserga/

Baserga & Kisieleski (1962b) reported that Ehrlich acites tumours maintained in the lungs, and small intestinal crypt cells in mice, showed evidence of reutilization of the label on the fourth day after injection of tritiated thymidine.

Diderholm et al. (1962) made three daily injections of tritiated thymidine into mice and two days after the final injection grafted pieces of skin obtained from an unlabelled animal into the injected animals. Cells in the epidermis of the grafted skin were found to be labelled one week after grafting. They considered that the most probable source of the label was the leukocytes that accumulated around the graft.

Cutright & Baur (1967), reporting their studies on the reutilization of tritiated thymidine during cell renewal in the oral epithelia and cheek skin of the rat, stated that the interval between the injection of tritiated thymidine and the recognition of reutilization of the label in autoradiographs was dependant on the turnover time of the epithelia. Epithelia with short turnover times showed evidence of reutilization of the label after a shorter interval than those with long turnover times. They implied that the local reutilization of the label was the most prominent one in the squamous stratified epithelia.

While the reutilization of labelled DNA would prove to be a source of error in long term studies of cell proliferation using labelled thymidine, such phenomena are of no serious consequence in most studies of cell proliferation which last for one or two days.

The Turnover and Repair of DNA:

It has long been thought that DNA is an extremely stable molecule and that under normal circumstances metabolism of DNA is associated with cell division, so that DNA synthesising cells are destined to divide with the exception of those undergoing polyploidy. However, in recent years Pelc and co-workers/

co-workers (Pelc, 1958; 1964) have put forward experimental evidence to suggest that in certain tissues there are cells that synthesise DNA at the normal rate, or at a very low rate, but do not proceed to divide. They concluded that such synthesis which is normally seen in a number of the so-called non-dividing tissues, was as a result of a metabolic turnover or repair of DNA.

Although there has been dispute regarding the metabolic turnover of DNA (Messier & Leblond, 1960; Gall & Johnson, 1960), such cells were not found to be present in fast renewing epithelia by Pelc and co-workers, and it seems reasonable to ignore the possibility of an error introduced by such turnover of DNA on studies of cell proliferation in squamous stratified epithelia.

Autoradiography

Autoradiography is the technique by which the presence of radioactivity in a particular area is recognised by the silver grains or tracts that are produced by the radioactive emission on a "photographic" emulsion, and it has been popularly used in the recognition of labelled cells in studies of cell proliferation. The advent of the techniques of autoradiography has also made valuable contributions towards the study of the sites of cellular metabolism. As for histology, C.P. Leblond (1965) remarked that autoradiography had introduced a fourth dimension, viz. time.

Phenomena that amount to what we presently call autoradiography were observed a hundred years ago by Niepce et St. Victor in 1867, who published an account of the blackening of emulsions of silver chloride and iodide by Uranium salts. He interpreted the results in terms of luminescence. His experiments were repeated and extended by Henri Becquerel in 1896 (Rogers, 1967). It is interesting to note that the "phenomenon of autoradiography" was observed even before the discovery of radioactivity.

Although it was London who made the first autoradiograph from biological material in 1904, the application of autoradiography for the detection of radioactivity in histological specimens was introduced and improved on by Lacassagne and co-workers in 1924 (Boyd, 1955). It was also Lacassagne who introduced the term autoradiography. However, nowadays the term radioautography has also been used by some workers. Boyd (1955), considering the pros and cons in relation to these two terms, was in favour of the term autoradiography. Baserga (1967) further suggested that the term autoradiography introduced by Lacassagne should be retained as a mark of tribute to his pioneering efforts in the field. While the opinion regarding terminology still remains divided, the term autoradiography will be used in preference to the term radioautography in this work.

While/

While autoradiographs were prepared by the apposition of photographic plates against the tissue specimens or histological sections, and the processed autoradiographs and the stained sections were studied separately by the earlier workers, the use of molten emulsion by Belanger & Leblond in 1946 made it possible to observe the pattern of radioactivity shown in the autoradiographs and the histology of the tissues simultaneously. Belanger & Leblond painted the emulsion melted down from photographic plates on the tissue sections with a fine brush. Arnold (1954) adapted the same method with nuclear emulsions. Joftes & Warren (1955) introduced the dipping method in which the slide was dipped once into the molten emulsion. The dipping technique was later improved by Kopriwa & Leblond (1962) and has been widely used with the present day nuclear emulsions. In the meantime, Pelc (1947) and Doniah & Pelc (1950) introduced the stripping film technique. The stripping film consists of a uniform layer of nuclear emulsion on a gelatin base mounted on glass plates. The technique involves the cutting of an appropriate piece of film, stripping it from the plate, floating it on water and then picking it on the slide with the emulsion surface in contact with the tissue section. Both the dipping technique and the stripping film technique have been widely used.

The first electron microscopic autoradiographs were prepared by Liquier-Milward (1956), and since then the techniques have been much improved to obtain higher resolutions.

The principles, techniques, and the applications of autoradiography have been adequately dealt with by Boyd (1955) and Rogers (1967).

Autoradiography in the Study of Cell Proliferation:

Under pulse labelling conditions in vitro or in vivo, the number of cells incorporating tritiated thymidine will be those that are in the S-phase of the cell cycle. These cells will have silver grains overlying their nuclei in autoradiographs, and/

and are referred to as labelled cells. The fraction of the number of labelled cells to the total number of cells in a cell population has been variously referred to as labelling index, radioactive index, ^{3}H index, and thymidine index (Baserga & Kisieleski, 1962a). The term labelling index will be used in this work in preference to the others.

The labelling index is sometimes considered as measure of the proliferative potential of a particular cell population (Cronkite et al., 1959). As already pointed out for the mitotic index, the labelling index is merely a measure of the number of cells that were in the S-phase at the time of pulse labelling, whereas the proliferative potential of the cells would depend on the rates of transit of these cells across the S-phase. Hence the rate of DNA synthesis becomes a more appropriate measure of cell proliferation. The rate of DNA synthesis is defined as the number of cells entering or leaving the S-phase per unit time (i.e. number of cells in DNA synthesis/duration of DNA synthesis).

Estimation of the Duration of the Cell Cycle and its Phases:

The pulse chase method; This is about the best method available for the estimation of the duration of the cell cycle and its phases. In principle it consists of pulse-labelling the cells present in the S-phase at a particular time by the injection of tritiated thymidine, and then observing their arrival in yet another compartment, viz. mitosis. The rise and fall of the fraction or percentage of labelled mitosis/mitosis as a function of the time after the injection of the label will depend on the rate at which the cells traverse the cell cycle, and hence the duration of the various phases of the cell cycle can be estimated from such a plot.

In the case of the ideal situation where all the cells would have exactly the same rates of transit, then no labelled mitosis will be seen for a period of time equal to the duration of the G_2 phase (T_{G2}) following a pulse label. Thereafter/

Thereafter as more and more labelled cells are beginning to divide, the percentage of labelled mitosis/mitosis would rise steeply to reach 100%. It would then stay at this level for a period of time equal to the duration of the S-phase (T_S), and then decline with same gradient as that for the rise to become zero when all the labelled cells have passed through mitosis. The fraction of labelled cells in a steady state system would then have doubled by this time. The labelled cells have now entered the G_1 phase, and if they are observed for sufficient length of time, labelled mitosis will again be observed after a period corresponding to the duration of G_1 phase, plus duration of S-phase, plus duration of G_2 phase ($T_{G1} + T_S + T_{G2}$). The percentage of labelled mitosis/mitosis will have a pattern of rise and fall similar to that for the first wave of labelled mitosis already described. The distance between any corresponding points on the first and second waves of labelled mitosis will then give an estimate of the duration of the cell cycle. The appearance of these regular waves of labelled mitosis would go on until the amount of label in the nuclei becomes too small to be recognised in autoradiographs owing to the halving of the label content at each division.

As variations between individuals is almost a characteristic of biological systems, the pattern of the waves of labelled mitosis described above becomes possible only in the theoretical situation. Variations in the transit rates of the individual cells of the cell population reduces the waves of labelled mitosis seen in actual practice to the form illustrated in figure 4 for most cell populations.

The main differences between the theoretical and the observed waves of labelled mitosis are:-

1) owing to the cells with faster rates of transit reaching mitosis earlier than the others, the time interval between labelling and the appearance of labelled mitosis is only an estimate of the minimum duration of T_{G2}.

2)/

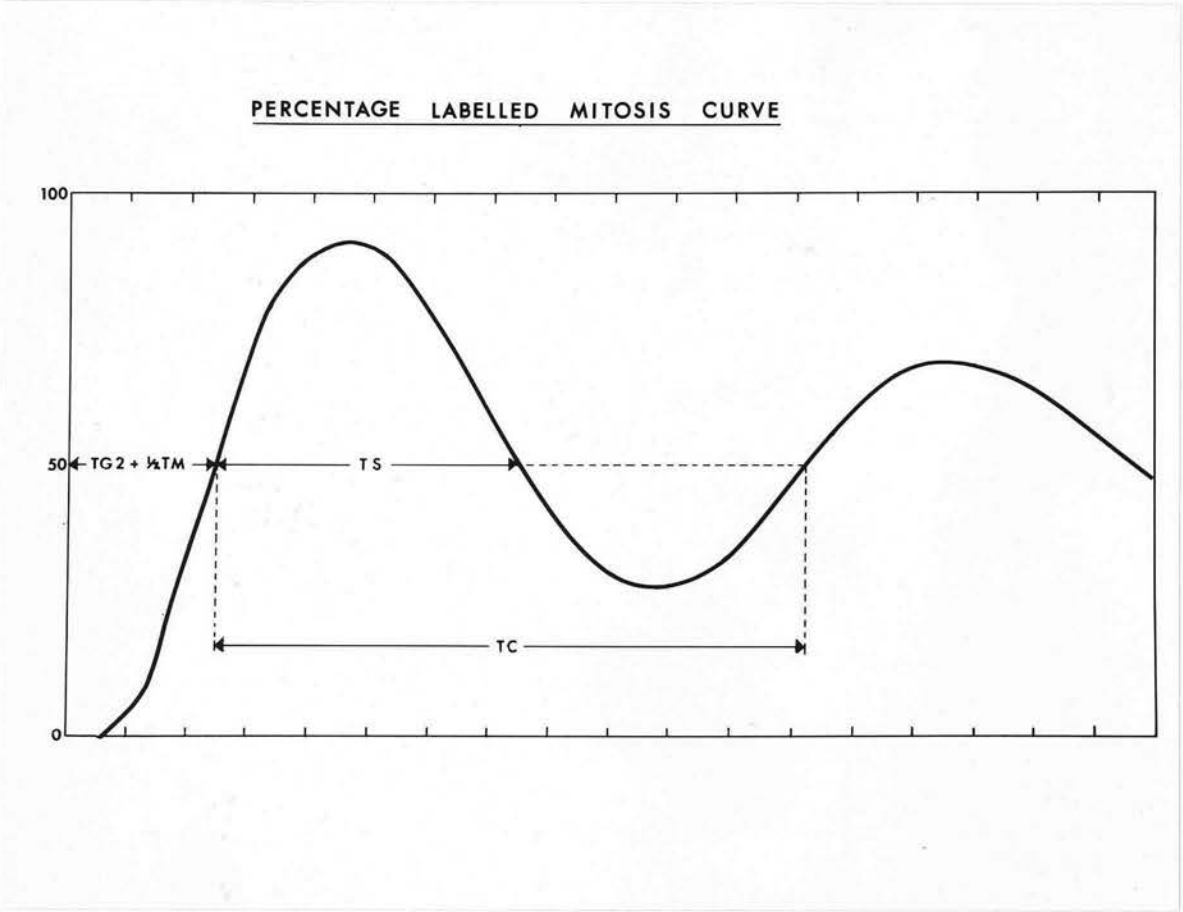

Fig. 4.

2) the percentage of labelled mitosis/mitosis does not normally reach 100% although in most cases it is usually more than 90%. This has been explained to result from the existence of false negative labelled mitoses as a result of the beta-particles of a few of the labelled mitotic figures at the depths of the sections not being registered in the emulsion (Johnson, 1960).

3) the gradient of the descending limb of the wave of labelled mitosis is usually less than that of the ascending limb.

4) the descending limb of the labelled mitosis wave does not reach zero especially in populations with short cell cycle durations.

5) in practice it is only possible to observe two and sometimes three waves of labelled mitosis, for the cells gradually get spread out in the cell cycle with time, as a result of the individual variations in the transit rates.

The estimates of the durations of the cell cycle and its phases could be obtained by the 50% intercepts on the waves of labelled mitosis as illustrated in Figure 4. Takahashi (1966), investigating the theoretical basis of this method, confirmed its reliability.

The First Wave of Labelled Mitosis, and Labelling and Mitotic Indices:

When it is not possible to obtain the second wave of labelled mitosis, the duration of the cell cycle can be estimated by the application of Wright's hypothesis.

Wright (1925), studying the duration of the various stages of mitosis in chick fibroblasts by direct visualization in vitro, and comparing them with frequency of occurrence of the various stages as seen in fixed and stained preparations, found that the ratio of the frequency of the different stages to their respective durations was fairly constant. He, therefore/

therefore, postulated that the number of cells present in a particular stage was proportional to the time spent in it. This rule of proportionality would also hold good for the rest of the phases of the cell cycle under steady state conditions, and has become known as Wright's hypothesis. Wright's hypothesis, however, does not hold good for true exponential, or tumour growth.

The duration of the cell cycle and mitosis can then be estimated from the following relationship:- number of labelled cells/duration of S-phase = total number of progenitor cells/duration of cell cycle = number of cells in mitosis/duration of mitosis.

As T_S and $T_{G2}+\frac{1}{2}T_M$ can be read from the first wave of labelled mitosis, and the labelling and mitotic indices can be easily determined, the duration of cell cycle and its phases may be estimated.

Double Labelling Technique:

This method consists of giving two injections of labelled thymidine separated by a short interval of time (about 1 hour). Either two injections of tritiated thymidine (Evensen,1962; Baserga & Lisco, 1963), or an injection of tritiated thymidine followed by another one with 14C thymidine (Baserga & Lisco, 1963; Wimber & Quastler, 1963) have been used. In the former, the additional cells found to be labelled by the second injection will be proportional to the interval between the first and second injections. In the latter, the number of cells in the population that have the tritium label only are the cells that have left the S-phase during the interval between the injections and hence is a measure of rate at which cells complete DNA synthesis. The cells labelled with tritium alone could be identified in autoradiographs by the fact that the silver grains will overly the nucleus unlike the case of 14_C labelled cells where there will be considerable scatter of the grains. While the injection of tritiated and 14_C thymidine allows study of cells labelled by the two injections in the same animal, the use of double injections of tritiated thymidine necessitates the use of/

of two groups of animals. Baserga & Lisco (1963), studying these two methods, found that there was considerable difficulty in distinguishing between tritium labelled and 14_C labelled cells in autoradiographs prepared from tissue sections, especially if a number of labelled cells were found close to each other, and hence suggested that for animal experiments using tissue sections for autoradiography, double injections with tritiated thymidine should be used.

The additional number of cells found to be labelled by the second injection of tritiated thymidine would then be the cells that have entered the S-phase during the interval between the injections, and the rate of DNA synthesis could then be estimated as the number of cells labelled by both injections minus the number of cells labelled by the first injection/interval between the two injections.

EXPERIMENT 1

Investigation of the cystostatic action of vinblastine sulphate[1] on the oral epithelia of hamsters.

Introduction:

As discussed in page 56 the Syrian hamster is resistant to the action of colchicine and colcemid, and hence these drugs could not be used in the study of mitotic activity in hamsters. Although it is known that vinblastine produces metaphase arrest *in vitro* (Palmer et al., 1960), as well as *in vivo* (Cutts, 1961; Cardinalli et al., 1961), there is not sufficient information to make use of this drug as a cytostatic agent in hamsters. Hence it was necessary to establish the suitability of this drug as a cytostatic agent.

Object of the Study:

The experiment was planned to determine:-

1) the time at which the drug takes effect after administration by the intra-peritoneal route,
2) whether the duration of this effect is longer than the proposed duration of the experiment,
3) the optimal dose for arrest of mitosis in hamsters,
4) whether the doses used have an effect on the rate of entry of cells into prophase.

Materials and Methods:

The Syrian hamsters used in this and the rest of the experiments were obtained from the same source[2]. Each hamster was housed in a separate cage and food and water were available *ad libitum*. They were fed on standard laboratory pellets and a mixture of brown bread and milk. The animals were not disturbed except during feeding and cleaning and were exposed to daylight. The conditions of housing and feeding were kept the same throughout the whole investigation.

Thirty/

1 Eli-Lilly & Co., Basingstoke, England.

2 Mr. D.I. Roberts, Coombehurst Prep. School, Coombehurst Drive, Basingstoke.

Thirty male golden hamsters of 4-5 months of age were divided into four groups, one control group and three experimental groups. Groups 2, 3 and 4 received 1 mg., 2 mg., and 4 mg. vinblastine per kg. body weight respectively. The drug was injected intra-peritoneally as an 0.1 gm.% solution. The controls received comparable volumes of distilled water. All the injections were given within 20 minutes and the control and experimental animals were sacrificed by cervical fracture from ½ to 8 hours after injection as shown below:-

Interval between injection and sacrifice	Gr.1	Gr.2	Gr.3	Gr.4
½ hour	1	2	0	2
1 hour	0	2	2	2
2 hours	1	2	2	0
4 hours	1	2	2	2
8 hours	1	2	2	2
	4	10	8	8

The cheek pouches, palate and tongue were excised, fixed in 10% buffered formalin, and the tissues were bisected along the midline before processing. Paraffin sections 6 microns in thickness were prepared at a distance of about 30 microns from each other and stained with haematoxlyn and eosin or Haematoxlyn and tartrasine. The areas studied were the epithelia of the cheek pouch, hard palate and ventral surface of the tongue.

Mitotic Counts:

Five histological sections from each tissue in each animal were studied. The number of mitosis in relation to 1000 basal cells were counted from each section, and mitotic activity was expressed as the number of dividing cells per 100 basal cells. In the epithelium of the tongue and palate suprabasal mitotic figures were also included in this count. The mitotic figures were classified according to their stage, and were recorded as prophase/

prophase, metaphase, or ana-telophase. The disappearance of the ana-telophases from the tissues was taken as the criterion for the effective block of mitosis at the metaphase stage.

Analysis of Data:

Regression lines were fitted for the percentage metaphases/basal cells over time by the method of least squares in each of the three tissues for each dose. These regression lines were fitted through the origin ($y=bx$) for it was assumed that the injected agent did not interfere with cells already in the metaphase stage so that the number of arrested metaphases at the time of injection becomes zero. The slopes (b) and their standard errors were estimated using the formulae given in appendix 1 B.

If the injected agent had significantly altered the rate of entry of cells into prophase (mitosis), or the duration of prophase, then the number of cells in prophase in the different experimental groups would be correspondingly affected. If, however, vinblastine did not exert its effect other than at the metaphase stage, the percentage of prophase/basal cells would not be significantly different from each other in the control and experimental groups. This was tested by an analysis of variance. As the distribution of the number of animals in the two-factor classification (dose and time) was not uniform, i.e. the same number of animals were not sacrificed each time in the different groups, the data cannot be analysed by a straightforward analysis of variance. Hence the method of analysis for non-orthogonal data was used (Kempthorne, 1967) and the data was analysed at the Regional Computing Centre, Edinburgh, using a programme available in the A.R.C. Unit of Statistics of the University of Edinburgh.

Observations/

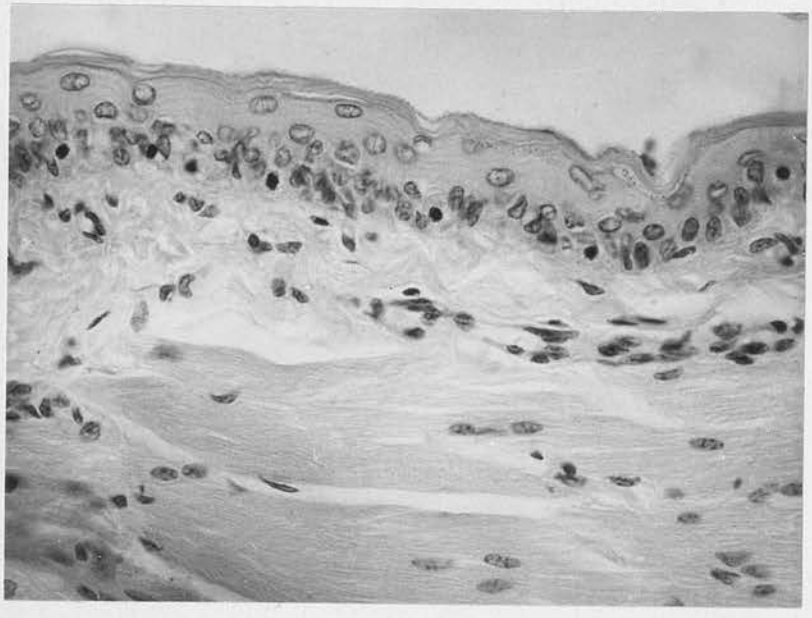

Fig. 5.

Cheek pouch of hamster 8 hours after injection of vinblastine sulphate.

H. & E. X63.

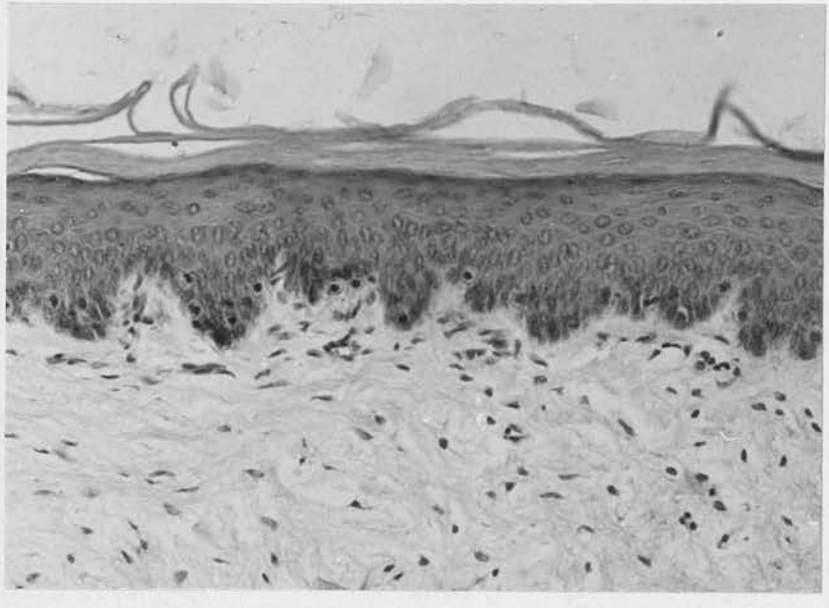

Fig. 6.

Hard palate of hamster 8 hours after injection of vinblastine sulphate.

H. & E. X40.

Observations:

The percentages of each of the phases of mitosis observed in each tissue of the control and experimental animals sacrificed over ½ to 8 hours after injection of vinblastine is shown in Appendix 1 A. Examination of these tables shows an increase in the percentage of metaphases/basal cells in the experimental groups with increase in time after injection of vinblastine. Two experimental animals in the 8 hour group showed only a slight increase in the number of metaphases and were excluded from the study. The reason for the absence of metaphase block may be that the drug was accidentally introduced into the lumen of the gut during intra-peritoneal injections. The increase in the percentage of metaphases/ basal cells over time is greater in animals receiving 2 mg. and 4 mg. of vinblastine per Kg. body weight than those receiving 1 mg. per Kg. body weight.

While ana-telophases continued to be present in the tissues of animals injected with 1 mg. or 2 mg. per Kg. body weight over all time groups, they were not present in the 4 mg. group from 1 hour after injection with the exception of one animal which showed 0.06% ana-telophases in the tongue epithelium 1 hour after injection. The metaphase block produced by vinblastine in the epithelia of the pouch, palate and tongue of the 4 mg. group is illustrated in figures 5, 6 and 7. The slopes ± standard error of increase in the percentage metaphases/basal cells is shown below.

	Pouch	Palate	Tongue
Group II	0.34 ± 0.09	2.32 ± 0.17	1.13 ± 0.31
Group III	0.79 ± 0.18	3.09 ± 0.36	2.57 ± 0.58
Group IV	1.02 ± 0.10	2.99 ± 0.23	3.02 ± 0.30

Analysis/

Fig. 7.

Ventral surface of hamster tongue 8 hours after injection of vinblastine sulphate.

H. & E. X40.

Analysis of Variance:

The results of the analysis of variance of the percentage prophase/basal cells in the different groups show that there are no significant differences between the various time and dose groups at the 95% level of confidence. The analysis of variance tables are shown in Appendix I C.

Discussion:

While vinblastine is capable of blocking cells in mitosis at the metaphase stage, this block is only partial with doses of 1-2 mg. per Kgm. body weight. The drug when injected in a dose of 4 mg. per Kg. body weight appears to bring about an "effective block" of the mitosis in the oral tissues of the hamster that were studied, and hence this appears to be the desirable dose. Although the slopes of the increase of percentage metaphases/basal cells with time in the 2 mg. and 4 mg. groups were not significantly different from each other (Appendix 1 D). The 4 mg. group showed higher values for the slopes except in the palate where the reverse was found. This very small difference is probably a result of random variation, especially in view of the small and unequal number of animals in the two groups. Larger numbers of animals were not used in this study for the original purpose of the experiment was to investigate the feasibility of vinblastine as a cytostatic agent, and not to obtain the best estimate of the mitotic rates (slopes).

Onset and Duration of the Cytostatic Effect:

Vinblastine appears to take maximal effect between $\frac{1}{2}$ and 1 hour after injection as assessed by the disappearance of ana-telophases from the tissues studied. Even though the effect of the drug persisted at least up to 8 hours after injection, the percentage arrested metaphases/basal cells observed in the 8 hour group was lower than would have been expected on the basis/

basis of the values obtained during the first few hours after injection. Similar observations have also been made with increasing time after injection of colchicine (Hooper, 1961; Blenkensopp, 1967). Hooper attributed this to two things; firstly, to a few cells escaping the metaphase block as a result of the decreasing concentration of the drug after a few hours, and secondly, to the degeneration and disappearance of a few of the arrested metaphases. Another possibility in this experiment is variation in the rate of metaphase accumulation due to fluctuations in the rate of mitosis during the 8 hour experimental period (9.30 a.m. - 5.30 p.m.). While Brown and Berry (1968) have demonstrated an increase in the mitotic index of the hamster cheek pouch from 8 a.m. to 12 noon and a significant drop between 12 noon and 8 p.m., Bullough & Lawrence (1966) have shown that not only the mitotic index but also the rate and duration of mitosis are variable during the 24 hour period. Hence it is possible that the mitotic rate is lower during the afternoon than the forenoon so that the observed value for the percentage arrested metaphases with increasing time after injection of vinblastine is lower than that predicted from the increase during the forenoon. It, therefore, appeared advisable to plan the duration of the experiment to be within 4 - 6 hours.

Mechanism of Mitotic Arrest:

Although the exact mechanism of the action of vinblastine is not known, the available evidence suggests that it acts as a spindle poison as originally suggested by Palmer et al. (1960).

Bruchovsky (1965), reporting that vinblastine did not affect the rate of progress of cells through the various phases of the cell cycle in vitro, stated that vinblastine must be exerting its effect at or immediately before mitosis.

Sachsenmaier/

Sachsenmaier (1966) stated that slime mould (Physarum polycephalum) was resistant to the action of colchicine and vinblastine, and attributed this to the intra-nuclear location of the mitotic spindle in the slime mould, and the absence of rupture of the nuclear membrane during mitosis which prevented the inhibitors reaching the mitotic spindle in effective concentrations. Thereby he implied that the site of action of these two agents was the mitotic spindle.

From his electron microscopic studies Krishan (1968) reported that the vinblastine-arrested mitoses were devoid of spindle microtubules, and that removal of the effect of vinblastine was soon followed by their re-appearance. He suggested that the mechanism of action of vinblastine was probably by depolymerisation of the spindle tubules or by the prevention of their synthesis and polymerisation.

Conclusions:

Vinblastine sulphate when injected in a dose of 4 mg. per Kg. body weight produces effective block of mitosis at the metaphase stage in the oral epithelia of hamsters without affecting the entry of cells into mitosis at least for 8 hours following intra-peritoneal injections.

EXPERIMENT 2.

Study of the cell cycle characteristics in the epithelium of the cheek pouch and palate of hamsters using tritiated thymidine autoradiography.

Materials and Methods:

Thirty male golden hamsters of 3 - 4 months of age were given intra-peritoneal injections of 1 micro-curie per gm. body weight of tritiated thymidine* (specific activity 5 curies/milli-mole), all the animals being injected within a period of 30 minutes. The injected animals were sacrificed by cervical fracture at 1-2 hourly intervals up to 24 hours as shown in Appendix 2 A, and thereafter one animal each at 30 hours, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days and 8 days, after the injection. Both cheek pouches and the palates were excised and fixed in 10% buffered formalin. The tissues were trimmed and washed in running water for 3-6 hours before processing, and paraffin sections 6 microns in thickness were prepared from the cheek pouches and the mid-line area of the palate at a distance of 30 microns from each other.

Autoradiographic Technique:

A pilot experiment was carried out to find out the suitable emulsion and exposure times for the autoradiographs under the conditions of the experiment. Kodak AR 10 stripping film, and Kodak NTB 2 and Ilford K5 nuclear emulsions were tested. There was no significant difference in the quality of the autoradiographs obtained with Kodak and Ilford nuclear emulsions. While the AR 10 film usually gives good results, the autoradiographs obtained in the pilot study were not satisfactory for reasons not known. Furthermore, it was possible to prepare almost twice the number of autoradiographs within a time period by the dipping-technique than by the stripping film technique. Hence/

* Radiochemical Centre, Amersham, England.

Hence the dipping-technique was used in this study. Ilford K5 emulsion was used, for the delivery time of this emulsion at the time of the study was much shorter than that for the Kodak NTB 2 emulsion. It was found that an exposure period of 2-3 weeks at 4°C. was satisfactory.

The autoradiographs were prepared by a modification of the method described by Rogers (1967) for Ilford nuclear emulsions. The nuclear emulsion used was diluted with distilled water (1:1) and kept melted at a temperature of about 43°C. The sections were deparaffinised, brought to water, and were dipped briefly into the emulsion. It was found advantageous to dip the slides in distilled water at 43°C. prior to coating with the emulsion, for the dipping of a warm slide favoured a more uniform flow of the emulsion and a less likelihood of getting air bubbles over the tissue sections. The other surface of the dipped slides were wiped and the coated slides were stood slanting at an oblique angle on aluminium racks, and dried in a gentle current of cold air. The dried slides were stored in light-proof plastic slide boxes* at 4°C. After suitable exposure (2-3 weeks) these slides were processed using Kodak D19 developer and Kodak Unifix fixer. The processing temperatures were kept at 18-20°C. and under these conditions a developing time of 2 minutes, and fixing time of 5 minutes, were found to be adequate. The autoradiographs were stained lightly with Erlich's Haematoxlyn and mounted in Canada balsam.

A number of autoradiographs were examined, and it was decided to consider a cell labelled provided it had about six silver grains overlying its nucleus. The background levels were very much lower than this, and any autoradiographs that showed considerable background levels were discarded.

Examination/

* Obtained from Arnold R. Howell Ltd., London, N.W.6.

Examination of Autoradiographs

Labelled mitosis: The autoradiographs were examined at a magnification of 100 x 10 and the percentage of labelled mitosis/total mitosis was computed for each animal by aggregating the scores obtained in about five autoradiographs. The total number of mitoses seen in each animal in the majority of cases was more than 100. A cell was considered to be in mitosis only after loss of nuclear membrane for there was some uncertainty associated with the recognition of labelled cells in early prophase. The labelled and unlabelled mitotic figures were classified as present in the basal or suprabasal layers, and plots of percentage labelled mitosis/total mitosis versus time were made.

Labelling and Mitotic Indices: The labelling and mitotic indices were determined by counting in most cases 2,000 - 5,000 basal cells. Only animals sacrificed within 12 hours after injection of tritiated thymidine were used for the determination of the labelling index in order to minimise errors introduced by the increase in the number of labelled cells as a result of these cells undergoing mitosis and doubling their numbers. Although corrections could be made for such an increase, it was assumed that the magnitude of this error would be similar to that due to the labelled cells at the depth of the tissue sections not being registered in the emulsion due to their short range of the beta particles and hence fail to be identified and counted.

Results:

The percentage of labelled mitosis/total mitosis in the epithelium of the cheek pouch and palate are shown in Appendix 2 A and B. Since there was no significant pattern observable in the percentage of labelled mitosis/total mitosis in animals killed after 24 hours, they were not included in this part.

The/

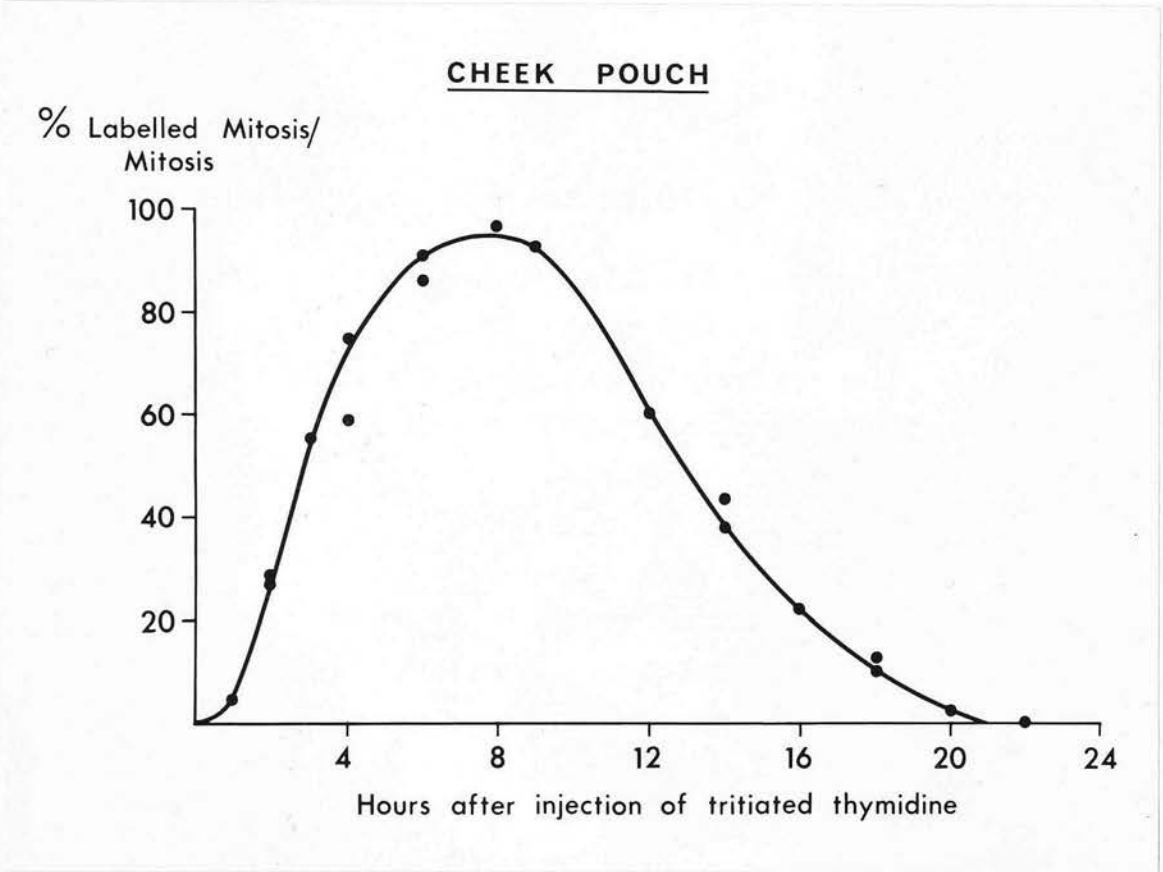
CHEEK POUCH
% Labelled Mitosis/ Mitosis
100
80
60
40
20
4
8
12
16
20
24
Hours after injection of tritiated thymidine

Fig. 8

The plot of the percentage labelled mitosis/total mitosis versus time for the cheek pouch epithelium is shown in Figure 8. This percentage increased gradually to reach 96% at 8 hours and then fell with a lesser gradient to reach zero only after 20 hours from the beginning of the experiment. The second wave of labelled mitosis was not discernible by the procedure used in this experiment. The estimates obtained through the 50% intercepts on this curve were as follows:

$$T_{G2} + \tfrac{1}{2} T_M = 2.8 \text{ hours}$$

$$T_S = 10.00 \text{ hours.}$$

Since the second wave of labelled mitosis did not materialise, duration of the cell cycle (T_C), duration of the G_1 phase (T_{G1}), duration of the G_2 phase (T_{G2}), and the duration of mitosis (T_M) were estimated by the application of Wright's hypothesis. The mitotic and labelling indices for the epithelium of the cheek pouch is shown in Appendix 2 A and their mean $\pm$ 1 standard error were found to be 1.08 $\pm$ 0.04% and 6.10 $\pm$ 0.33% respectively. The estimate of the duration of the cell cycle and its phases was made as follows:

$$T_C = \frac{T_S \times N_C}{N_S},$$ where N_C is the number of progenitor cells, and N_S is the number of labelled cells.

$$= \frac{10.00 \times 100}{6.10} \text{ hours}$$

$$= 163.9 \text{ hours}$$

Similarly, $$T_M = \frac{T_S \times N_M}{N_S}$$

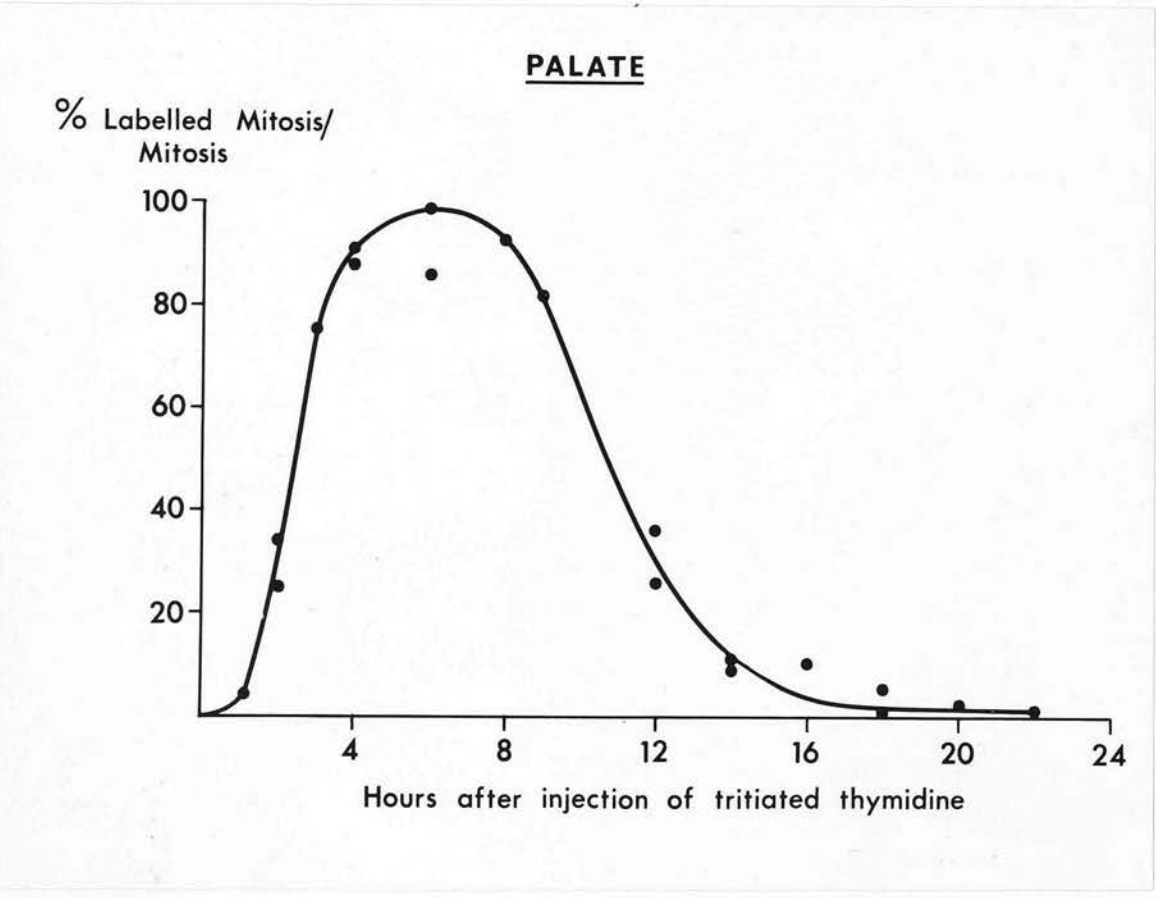
PALATE
% Labelled Mitosis/ Mitosis
100
80
60
40
20
4
8
12
16
20
24
Hours after injection of tritiated thymidine

Fig. 9.

$$= \frac{10.00 \times 1.08}{6.10} \text{ hours}$$

$$= 1.77 \text{ hours}$$

and $T_{G2} = (T_{G2} + \frac{1}{2} T_M) - \frac{1}{2} T_M$

$$= (2.8 - 0.88) \text{ hours}$$

$$= 1.92 \text{ hours.}$$

$$T_{G1} = T_C - (T_S + T_{G2} + T_M)$$

$$= 163.9 - (10.00 + 1.92 + 1.77)$$

$$= 150 \text{ hours.}$$

The percentage of labelled mitosis/total mitosis in the palate in the animals sacrificed at various times after injection is shown in Appendix 2 B.

The plots of percentage of labelled mitosis/total mitosis versus time is illustrated in Figure 9. This percentage increased gradually to 98% at 6 hours, and decreased to reach zero after 18 hours. The examination of the location of the mitoses and the labelled cells in the palate indicated that about 35% of the mitotic figures and labelled cells were present in the suprabasal layers of the palate. Figure 10 illustrates the presence of labelled cells only in the basal layer of the cheek pouch epithelium, while Figures 11 and 12 show the presence of labelled cells and mitotic figures in the suprabasal layers in the epithelium of the palate. Plots of labelled mitosis/total mitosis versus time based on the location of the mitotic figures, as in the basal layer or suprabasal layers, is illustrated in Figure 13. The values obtained through the 50% intercepts are as follows:

Basal/

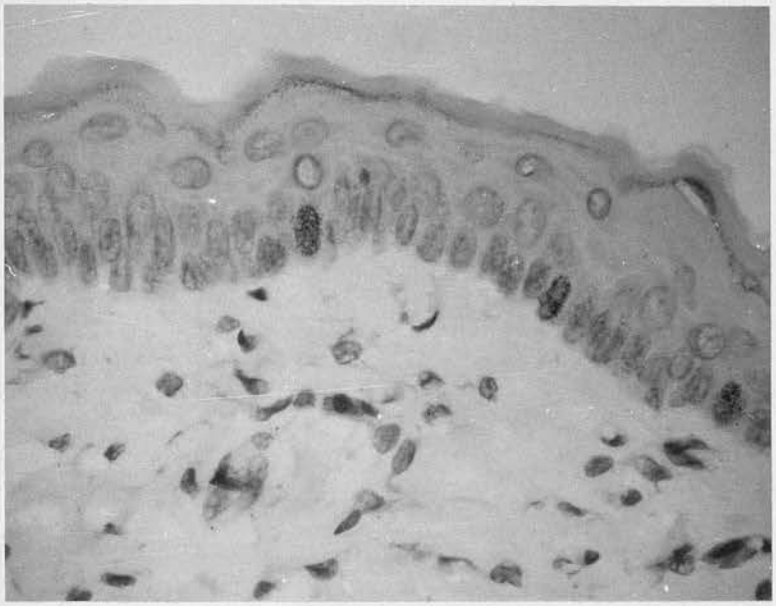

Fig. IO.

Autoradiograph of cheek pouch of hamster sacrificed I hour after the injection of tritiated thymidine.

Haem. XIOO

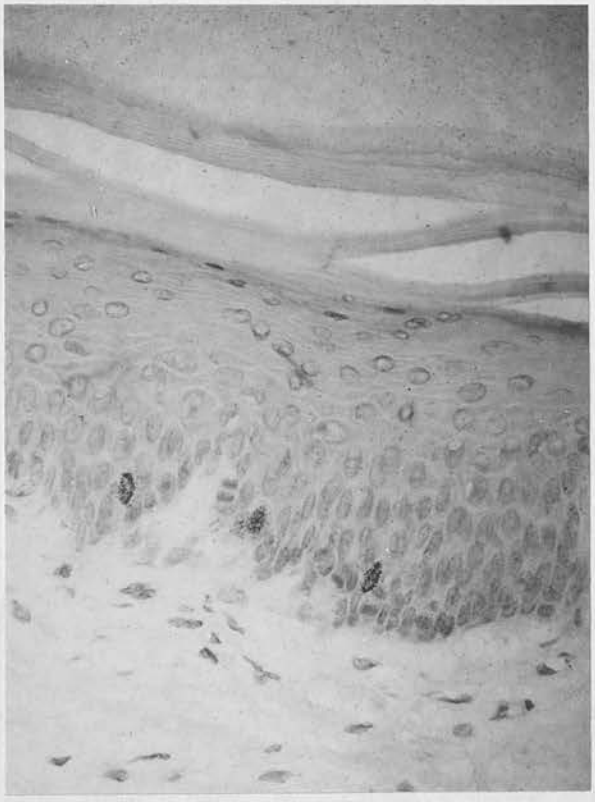

Fig. II.

Autoradiograph of the hard palate of hamster sacrificed I hour after the injection of tritiated thymidine.

Haem. XIOO.

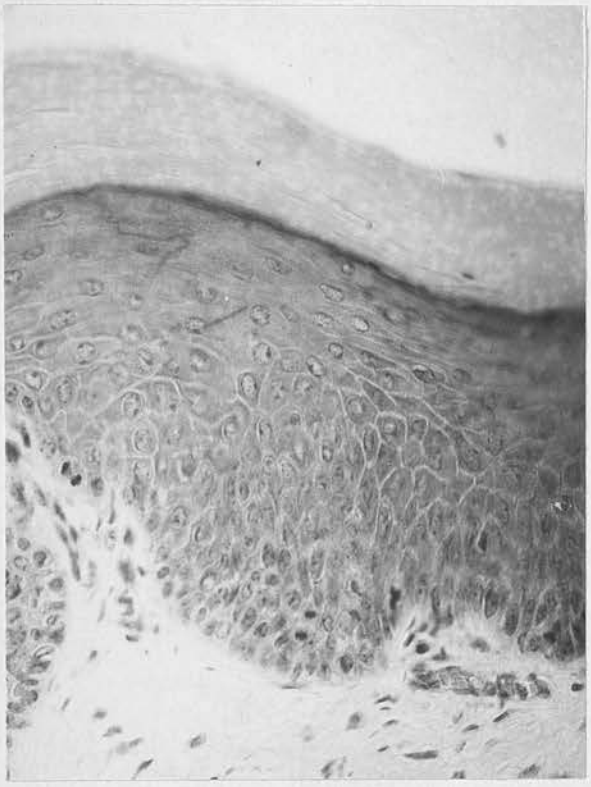

Fig. I2.

Hard palate of hamster.

H. & E. XIOO.

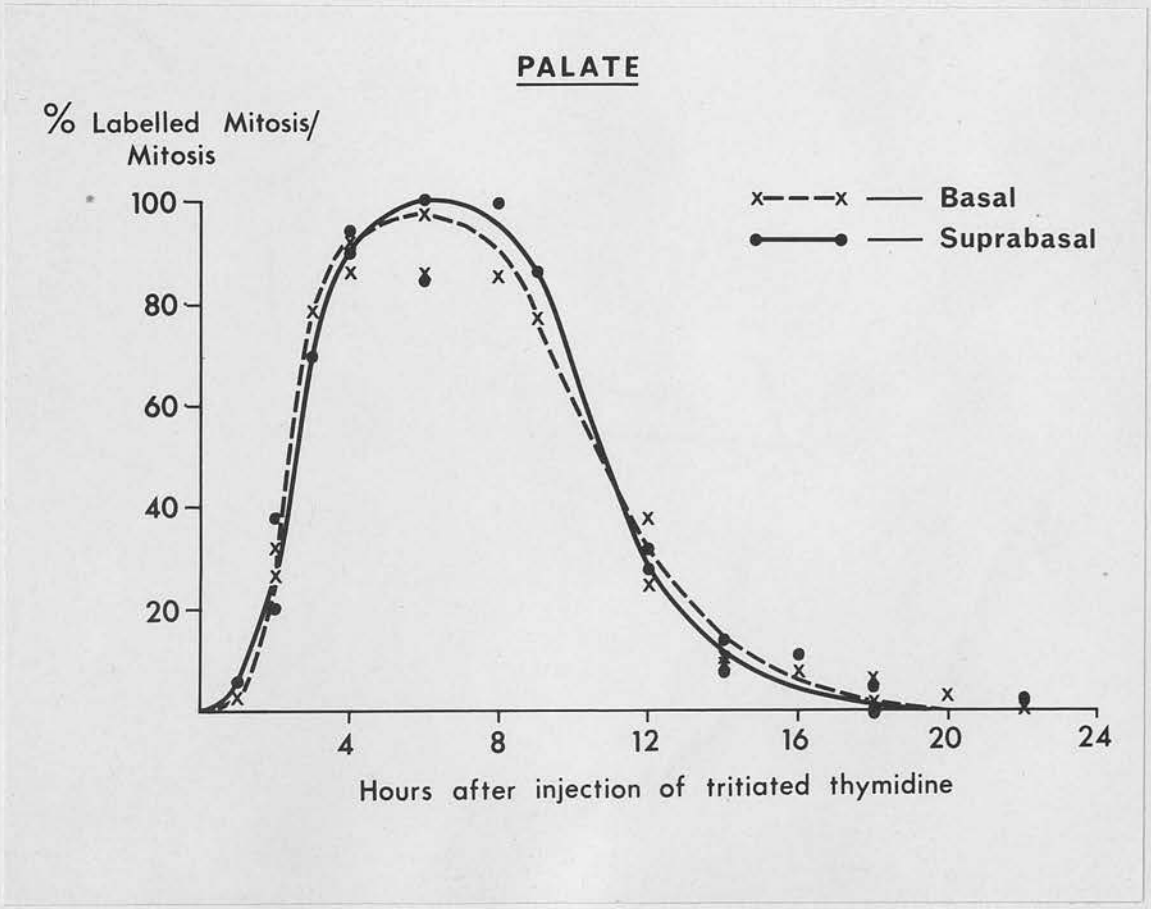

Fig. I3.

Basal cells

T_S = 8.26 hours.

$T_{G1} + \frac{1}{2} T_M$ = 2.53 hours.

Suprabasal cells

T_S = 8.26 hours.

$T_{G1} + \frac{1}{2} T_M$ = 2.66 hours.

The details of the mitotic and labelling indices for the basal layer of the palate are shown in Appendix 2 B and the mean ± 1 S.E. were found to be 2.19 ± 0.12% and 11.11 ± 0.72% of the basal cells respectively. The cell cycle durations for the basal cells were estimated using Wright's hypothesis as follows:

$$T_C = \frac{Ts \times Nc}{Ns}$$

$$= \frac{8.26 \times 100}{11.11}$$

$$= 74.3 \text{ hours.}$$

$$T_M = \frac{Ts \times N_M}{N_S}$$

$$= \frac{8.26 \times 2.19}{11.11}$$

$$= 1.62 \text{ hours.}$$

$$T_{G2} = (T_{G2} + \tfrac{1}{2} T_M) - \tfrac{1}{2} T_M$$

$$= (2.53 - 0.81)$$

$$= 1.72 \text{ hours.}$$

$$T_{G1} = T_C - (T_S + T_{G2} + T_M)$$

$$= 74.3 - (8.26 + 1.72 + 1.62)$$

$$= 62.7 \text{ hours.}$$

Discussion

While the mitotic figures and labelled cells were almost exclusively present in the basal layer of the epithelium of the cheek pouch, about 35% of them were present in the suprabasal layers of the palatal epithelium. The presence of a considerable proportion of the total number of mitotic figure and labelled cells in the suprabasal layers has also been reported for the palatal epithelium of rats and mice (Meyer et al., 1960; Sharav & Massler, 1967).

Plots of the percentage of labelled mitosis/total mitosis versus time for the basal and suprabasal mitotic figures showed that these two curves are not significantly different from each other with regard to their shape or the values obtained through their 50% intercepts. Hence these two groups of cells appear to have the same rates of transit through the cell cycle. There are two possibilities regarding the origin of the suprabasal mitotic figures and labelled cells, one is that there is a sub-population of progenitor cells present in the suprabasal layers of the palatal epithelium, and the other is that these are cells that have been pushed out of the basal layer after they have taken the decision to divide, as it is accepted that migration of cells from the basal layer is due to crowding produced by the addition of new cells (Leblond et al., 1964). However, the former view seems more plausible for the following reasons:-

1) Marques-Pereira & Leblond (1965), studying the proliferation and migration of cells in the oesophageal epithelium of rats where the stem cells are confined to the basal layer, found that while only about 0.4% of the labelled basal cells were seen to migrate out of the basal layer between 6 - 12 hours after the injection of tritiated thymidine, this increased to a steady rate of 1.2% per hour thereafter. They stated that this difference in the rates before and after 12 hours was due to the fact that the cells in DNA synthesis and in mitosis were migrating out of the basal layer only exceptionally/

exceptionally, and once these cells complete division they had an equal chance of moving out of the basal layer.

Brown & Oliver (1968), studying the migration of labelled cells in the hamster cheek pouch, observed that there was no significant migration of labelled cells out of the basal layer for about 20 hours from the time of injection of tritiated thymidine. Observations in the hamster cheek pouch in the present study are in agreement with those of Brown & Oliver. The fact that the time after which the migration of labelled cells become significant in the epithelia of the oesophagus and the cheek pouch is the same as the time taken for the first wave of labelled mitosis to rise and fall in these two, further supports the suggestion of Marques-Pereira & Leblond (1965).

Hence the presence of 35% of labelled cells and mitotic figures in the suprabasal layers of the palate cannot be accounted in terms of mere migration of these from the basal layer.

2) Sharav & Massler (1967), studying the age changes in the oral epithelia of rats, found that while there was a considerable increase in the percentage of labelled basal cells in the palate between 9 and 27 months of age, there was an accompanied decrease in the fraction of suprabasal labelled cells. Had these suprabasal labelled cells been originating from the basal layer, and not cells that were inherently present in the suprabasal layers, then the fraction of these cells would have remained the same or increased when there was an increase in the percentage of labelled basal cells. The observations of Sharav & Massler (1967), in fact, show the opposite and their conclusion that the supra-basal labelled cells were from a suprabasal progenitor pool which was decreasing with age, seems justifiable.

3) In addition to the above arguments, the rise and fall of the percentage of suprabasal labelled mitosis/suprabasal mitosis to yield a regular wave, suggests that they form an independent sub-population of progenitor cells and are not randomly migrating labelled cells and mitotic figures from the basal layer.

For/

For the reasons put forward above, the duration of the cell cycle and its phases in the palate was estimated for the basal cells from the percentage of labelled cells and mitosis in the basal layer only. Although similarity in the first wave of labelled mitosis for basal and suprabasal cells suggests that the duration of the cell cycle is similar in the two, a direct estimate of the duration of the cell cycle in the suprabasal cells could not be obtained for the total number of progenitor cells in the suprabasal layers is not known.

The duration of the cell cycle in the epithelium of the cheek pouch was estimated as 163.9 hours. A direct measurement of this duration from the waves of labelled mitosis is not possible in epithelia of long generation time, for it is not practicable to obtain the second wave of labelled mitosis in these cases. Brown & Oliver (1968) have described a method for the estimation of the generation time of such epithelia based on the rates of migration of labelled cells out of the progenitor compartment. They estimated the duration of the cell cycle in the cheek pouch epithelium of albino hamsters as 130 hours, and claimed that this estimate was close to that obtained by another method (135 hours). The method of Brown & Oliver, however, is of limited application for it could be used only in epithelia where the progenitor cells are confined to the basal layer.

Reiskin & Berry (1968) estimated the duration of cell cycle in the cheek pouch epithelium of golden hamsters, using Wright's hypothesis, as 141.8 hours. The disparity between the estimates by Reiskin & Berry and that in the present study (163.9 hours) is due to the differences in the estimates of the duration of S-phase and the labelling indices in the two studies. While the duration of the S-phase was estimated as 9.9 hours, and 10 hours by Reiskin & Berry, and in the present study respectively, the labelling index was found to be 7% by Reiskin & Berry and 6.1% in the present study. Unfortunately small variations/

variations in the duration of S-phase as well as the labelling index brings about large differences in the duration of the cell cycle when estimated by the application of Wright's hypothesis.

The duration of the various phases of the cell cycle as estimated in the present study and by Reiskin & Berry (1968) is shown below:-

	T_S	T_{G2}	T_M	T_{G1}
Present study	10.0 hrs.	1.92 hrs.	1.77 hrs.	150 hrs.
Reiskin & Berry (1968)	9.9 hrs.	2.6 hrs.	1.6 hrs.	126.4 hrs.

The duration of the cell cycle in the epithelium of the hard palate is about 74.3 hours, in comparison to that of 163.9 hours in the cheek pouch epithelium. The duration of the S-phase, G2 phase, and mitosis in the palatal epithelium is shorter than the corresponding ones in the cheek pouch. However, statistical significance of these differences cannot be tested for it is not possible to obtain meaningful confidence limits of these values. The labelling and mitotic indices in the palatal epithelium are significantly higher than those in the epithelium of the cheek pouch (Appendix 2 C).

The mean rates of transit of the progenitor cells across the cell cycle in the epithelium of the pouch and palate are (100/163.9) 0.61% and (100/74.3) 1.35% respectively. However, the phase durations in the two epithelia do not simply vary as an inverse function of these rates, and the difference in the duration of the cell cycle in the cheek pouch and the palate is mainly due to a wide difference in the duration of the G1 phase in the two epithelia. The duration of the G1-phase in the cheek pouch is 150 hours, while that in the palate is only 62.7 hours. This finding further supports the view that the G1-phase is the phase of most variable duration.

Conclusions/

Conclusions

The duration of the cell cycle in the epithelium of the hamster cheek pouch is more than twice that in the palatal epithelium. While the progenitor cells are confined to the basal cell layer in the cheek pouch epithelium so that it would fit into a simple three-compartment renewal system, there is an additional sub-population of progenitor cells in the suprabasal layers of the palatal epithelium.

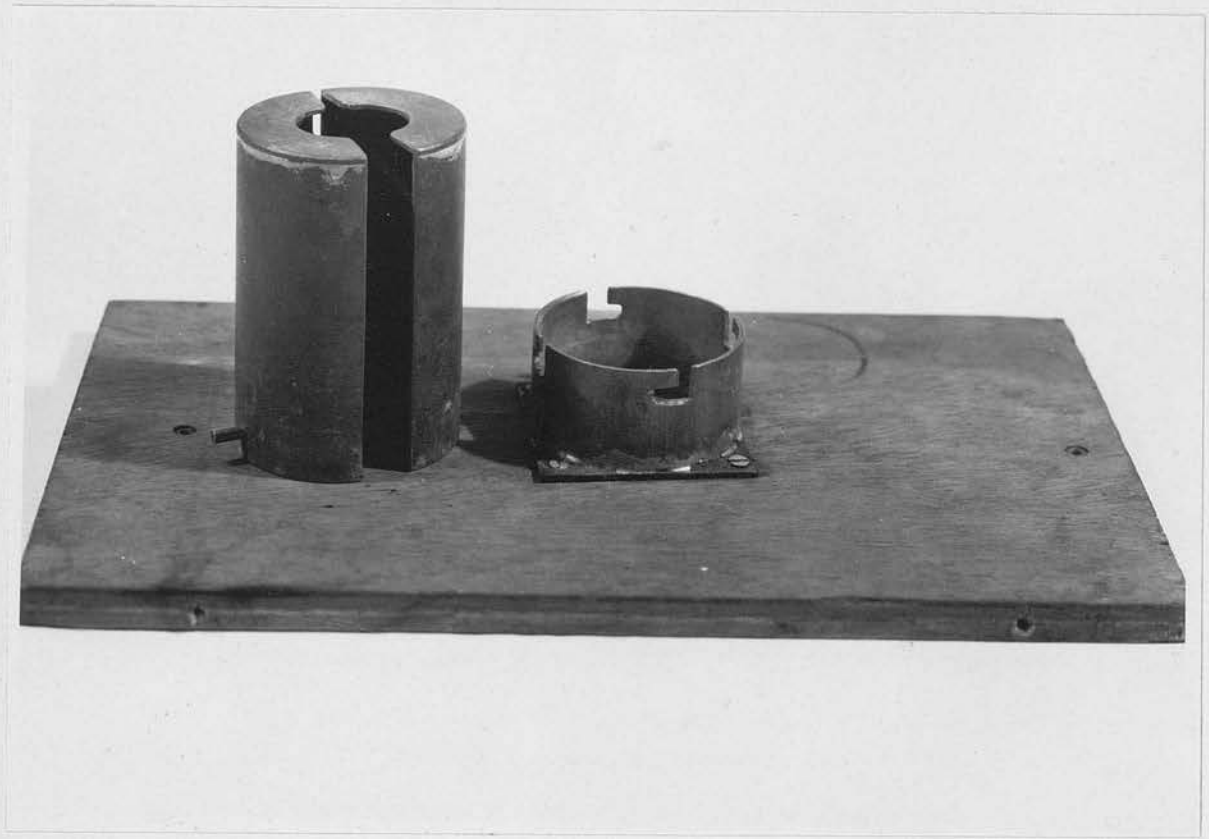

Fig. I4.

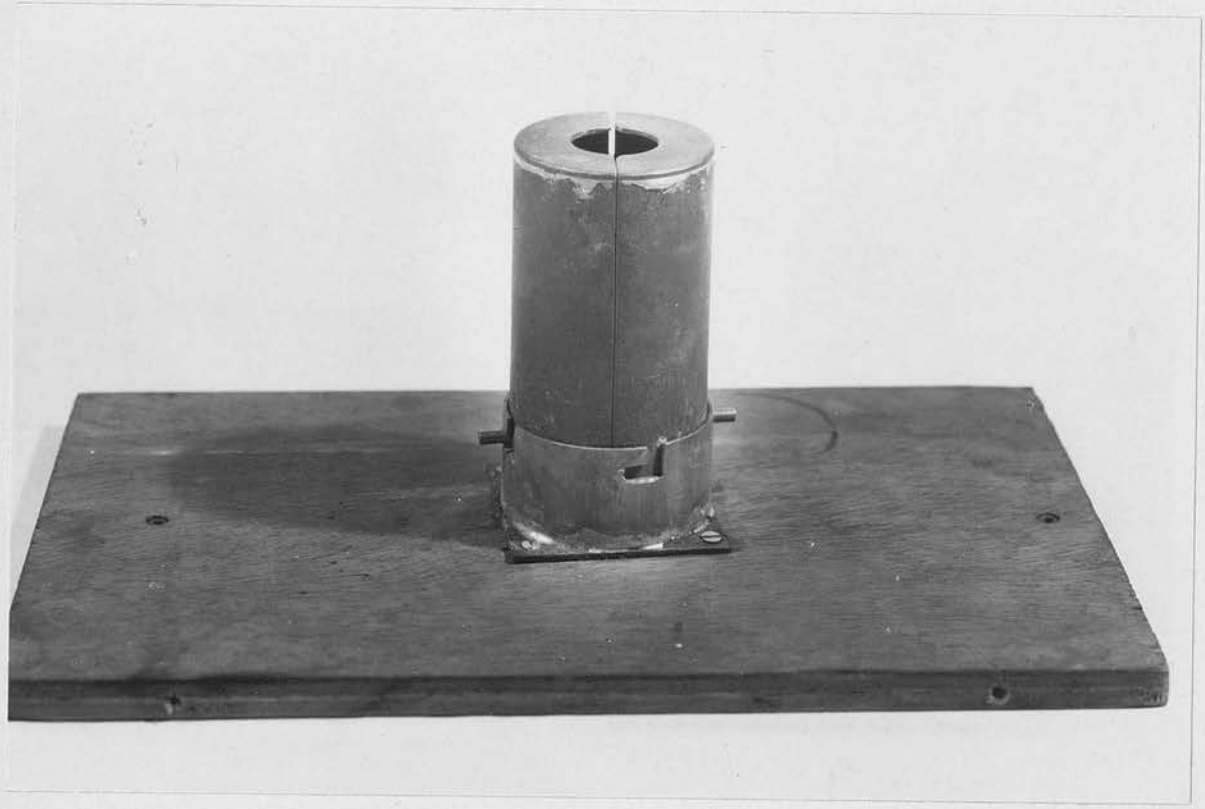

Fig. I5.

EXPERIMENT 3

Experimental induction of tumours in the Hamster cheek pouch by the topical applications of DMBA.

Introduction:

As already discussed in pages 3 and 7 , although the sequence of histological changes during hydrocarbon carcinogenesis of the hamster cheek pouch is the same irrespective of experimental conditions, the time taken for the development of tumours and the various intermediate stages depends on factors such as the potency of the carcinogen, nature of the solvent, concentration of the carcinogen, frequency of application, age and genetic background of the animal, etc. (Salley, 1954, 1957; Morris, 1961). Hence it was necessary to establish roughly the time taken for the development of the various stages during carcinogenesis of the hamster cheek pouch under the conditions that prevailed during the present study.

Objects of the Study:

The present experiment was planned to observe the time taken for the development of regular epithelial hyperplasia and tumours in the hamster cheek pouch under conditions of housing, feeding, carcinogen treatment, etc. in the present study.

Materials and Methods:

The right cheek pouches of 12 male hamsters of 3-4 months of age were painted with an 0.5% solution of DMBA in acetone three times weekly for 12 weeks. The animals were immobilised by using a variation of the apparatus described by Moss et al. (1965) (Figures 14 and 15). It consisted of a cylindrical metal capsule, and a wooden base on to which was attached a locking/

locking mechanism for the capsule. The cylindrical metal capsule consisted of two halves joined by a hinge mechanism which enabled the capsule to be opened and closed along its long axis. Each half of the capsule had a metal pin attached to its most convex part near its lower end. The lower end of the capsule was open while the upper end was closed except for the perforation at the centre.

Un-anaesthetised animals were manipulated into the partially opened capsule through its lower end, so that the head was projecting through the perforation in the upper end of the capsule. Once the animal was positioned in this manner the capsule was closed, seated on the base, and the pins of the capsule were locked into the L-shaped slots on the locking device. Once in this position the mouth of the animal was kept open by means of gauze or rubber loops around the upper and lower incisors by the assistant while the operator retracted the lateral wall of the cheek pouch with a blunt instrument. Excellent visibility and ease of operation were obtained by this method. As claimed by Moss et al. (1965) this method of immobilisation did not have any observable untoward effects on the animals.

The painting was carried out with a No. 4 camel hair brush using the wiped-brush method of Morris (1961). The carcinogen was applied to the cheek pouch mucosa by two to three clockwise sweeping movements of the brush starting and ending at the medial wall of the pouch. The cheek pouches were examined for gross changes before each painting. The carcinogen solution itself was stored in a dark bottle in order to minimise photo-oxidation, and was dispensed into a small container in appropriate quantities on each day of painting.

Since the object of the study was to establish the time taken for the development of epithelial hyperplasia and neoplasia under the conditions of the present experiment, no animals were sacrificed during the first two weeks of the painting. Animals were sacrificed by ether inhalation during the third, fifth, seventh., eighth, eleventh, twelfth, thirteenth/

Fig. I6.

Untreated (left) hamster cheek pouch.

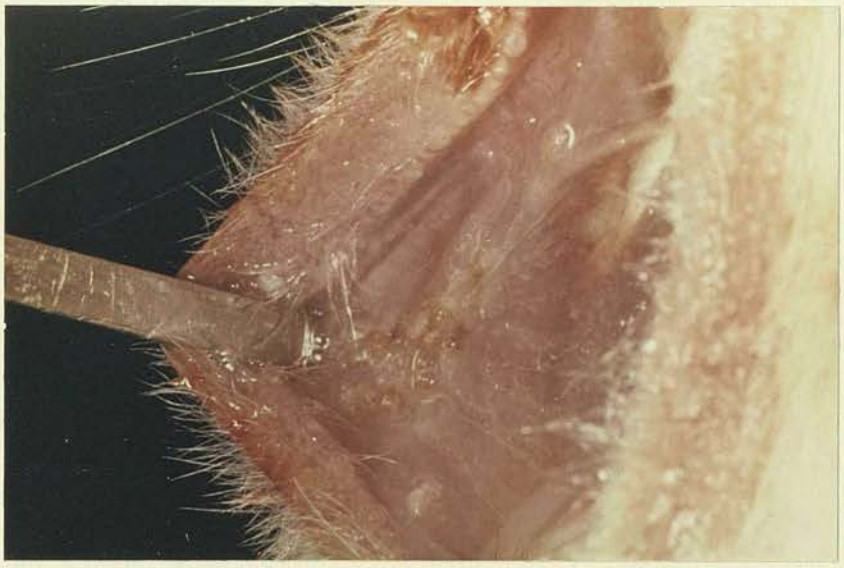

Fig. I7.

Hamster cheek pouch (right) after 5 weeks of carcinogen treatment.

thirteenth, fourteenth and fifteenth weeks of the experiment. One animal was sacrificed each week except during the thirteenth week when two were killed. Some of the treated pouches were photographed prior to excision for histology. The tissues were fixed in 10% buffered formalin, and paraffin sections 6 microns in thickness were prepared and stained with haematoxylin and eosin.

Observations:

Gross: There was marked redness of the painted pouches during the first week with gross ulceration being noticeable in most of the treated pouches by the second week of painting. By the end of the third week most of the ulcers appeared to have healed in spite of the continued painting, and in those animals with extensive ulceration the depth of the pouch was decreased probably due to proximal adhesions during healing.

After 5 weeks of painting the treated mucosa had become whitish and opaque, and did not have the glittering translucency seen in untreated mucosa. The gross appearance of untreated and treated cheek pouches at this stage is illustrated in Figures 16 and 17 respectively.

Tumours were first observed in two animals during the seventh week, and presented as nodular outgrowths of about 2 mm x 2 mm in size. Figure 18 shows one of these tumours one week after it was first observed. The other tumour continued to grow until it reached about 8 mm in diameter by the twelfth week when the animal was sacrificed. By the twelfth week all the surviving animals had developed tumours, and Figure 19 shows a cheek pouch which had developed multiple tumours after 12 weeks of carcinogen treatment. The average latent period for the development of tumours was 8 weeks, judged from the gross appearances.

Two out of the 12 experimental animals died during the experimental period.

Histological/

Fig. I8.

Hamster cheek pouch after 7 weeks of carcinogen treatment.

Fig. I9.

Hamster cheek pouch after I2 weeks of carcinogen treatment.

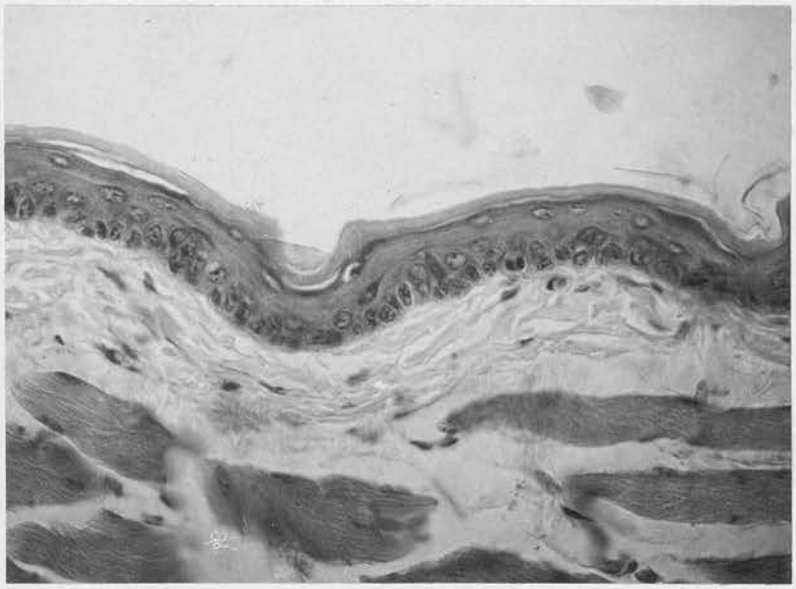

Fig. 20.

Normal hamster cheek pouch.

H. & E. X63.

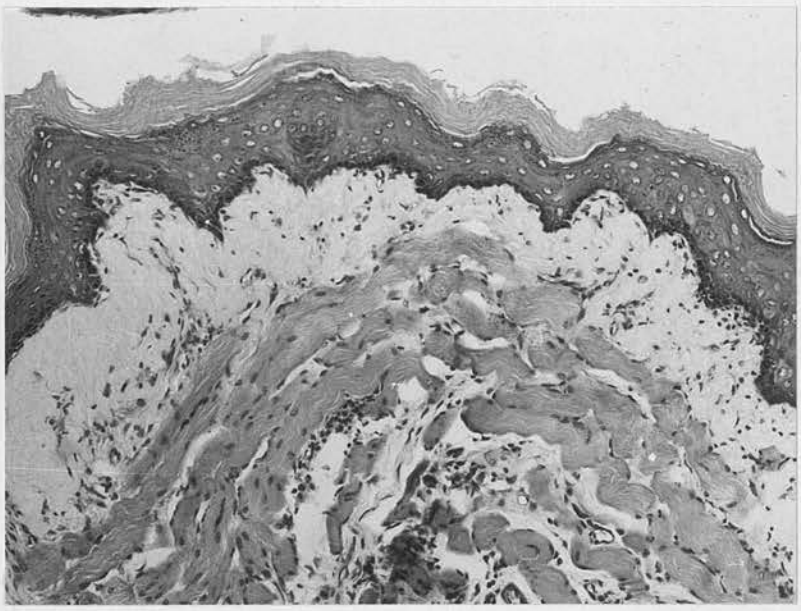

Fig. 21.

Hamster cheek pouch after 5 weeks of carcinogen treatment.

H. & E. X40.

Histological: The normal cheek pouch mucosa consisted of a thin keratinising stratified squamous epithelium consisting of about 3-4 cell layers in thickness and devoid of rete pegs. The sub-epithelial layers consisted of loose connective tissue beneath which were bundles of striated muscle (Figure 20).

The treated pouches excised during the third week of painting showed focal areas of chronic inflammation and ulceration, while the rest of the epithelium appeared thinner than that of normal cheek pouch, with masses of keratin in the lumen of the pouch. The characteristic feature of the treated mucosa excised during the fifth week of painting was the regular epithelial hyperplasia and hyperkeratosis (Figure 21). The basal layer of the epithelium had developed rete-pegs and there was an increase in the thickness and cellularity of the sub-epithelial connective tissue. While a great extent of the treated mucosa had the above appearance, there was also an ulcer (Figure 22) observable in the same pouch. The cheek pouch examined after 6 weeks of painting showed epithelial hyperplasia, hyperkeratosis, and areas of focal epithelial atypia.

All the pouches but one that were excised from the eighth week onwards had developed squamous cell carcinomas, and one such tumour is illustrated in Figure 23. The mucosa surrounding the tumours was hyperplastic and hyper-parakeratotic or hyper-orthokeratotic. The thickness of the epithelium was much greater than that observed earlier, and there was a number of highly dyskeratotic lesions (carcinoma in situ) with loss of stratification, epithelial atypia, nuclear hyperchromatism and increased keratinisation (Figure 24).

Discussion:

Although there is agreement about the various stages of development during the induction of tumours by the topical application of DMBA, the time taken for the development of these stages is not the same in the different investigations (Salley, 1957; Camilleri & Smith, 1965; Levij et al., 1967), and/

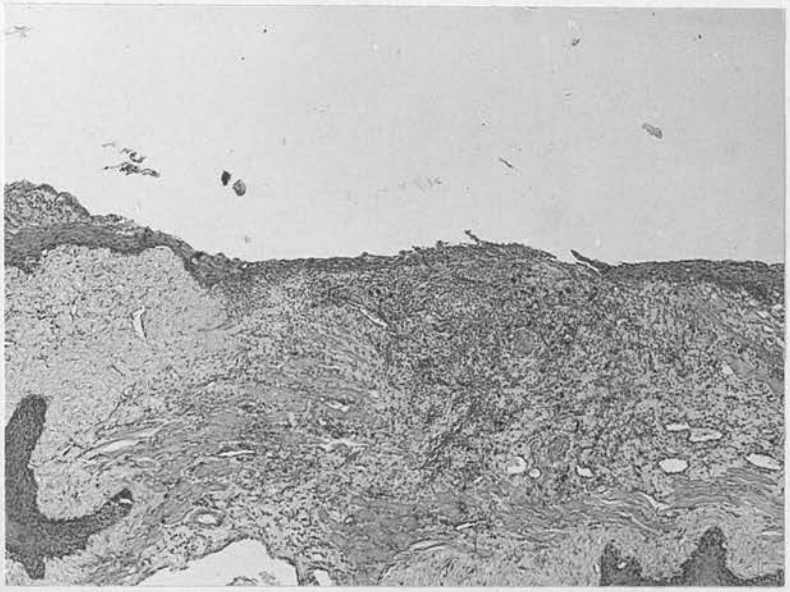

Fig. 22.

Ulcer in cheek pouch after 5 weeks of carcinogen treatment.

H. & E. X20.

Fig. 23.

Squamous cell carcinoma of hamster cheek pouch.

H. & E. X20.

Fig. 24.

Carcinoma in situ; same cheek pouch as Fig. 23.

H. & E. XI6.

and hence it was necessary to establish the approximate time taken for the development of epithelial hyperplasia and neoplasia under the experimental conditions of the present study.

The nature of the gross and histological changes observed in this study was essentially similar to those already described (Salley, 1957). Under conditions of the present study, five weeks of carcinogen treatment (15 applications) appeared to be optimal for the induction of regular epithelial hyperplasia. After the sixth week the treated pouches showed a greater tendency to develop epithelial atypia, that is dyskeratotic rather than a hyperplastic change.

A latent period of 8 weeks for the development of tumours observed in the present study is close to that observed by Salley (1954) who also used DMBA in acetone as the carcinogen. The nature of the solvent used to dissolve the carcinogen considerably influences the rapidity of the induction of tumours (page 20). Acetone was used as the solvent for DMBA in preference to benzene or mineral oil in this study for the former has a higher toxicity than acetone (Bradbury et al.,1941; Stowell & Cramer, 1942; Salley, 1954), and the latter is thought to be prone to produce digestive disturbances (Stormby & Wallenius, 1964). In addition, since mineral oil, when used as the solvent for DMBA, reduces the latent period for development of tumours to about 5 weeks (Salley, 1955), the rapidity of tissue changes might make it difficult to characterise the development of the intermediate stages during carcinogenesis in terms of the number of applications of the carcinogen that is required to induce them. With a less rapid induction of tumours, however, the transition from one stage of development to the other is more gradual, and therefore acetone was used as the solvent in the present study.

Conclusions/

Conclusions:

Under the conditions of the present experiment, topical applications of an 0.5% DMBA solution of DMBA in acetone three times weekly produced the following changes in the hamster cheek pouch:-

1) Gross ulceration of the pouches after one week of carcinogen treatment.

2) Regular epithelial hyperplasia by five weeks of carcinogen treatment.

3) Epithelial atypia from the seventh week onwards.

4) Gross tumours by the seventh week from the beginning of the experiment.

5) Squamous cell carcinomas from the eighth week onwards.

EXPERIMENT 4.

Cell Cycle Studies on Carcinogen induced Epithelial Hyperplasia of the hamster cheek pouch.

Materials and Methods:

The right cheek pouches of 30 male golden hamsters of 2-3 months of age were painted with an 0.5% solution of DMBA in acetone, three times weekly, for 5 weeks as already described (Experiment 3). The experiment consisted of two parts, one for the determination of the rate and duration of DNA synthesis (16 animals), and the other for the determination of the rate and duration of mitosis (14 animals). The animals were allocated into different groups at random at all stages of the experiment, and the experiments were performed at the end of the sixth week from the beginning of painting.

Part 1. Sixteen animals were allocated into two groups, each group consisting of 8 animals. Both groups were given intraperitoneal injections of 1 micro-curie per gm. body weight of tritiated thymidine (specific activity 5 curies/milli-mole) at 9.30 a.m. The animals in Group 1 were sacrificed one hour after injection, while those in Group 2 received a second injection of tritiated thymidine 1½ hours after the first injection, and were sacrificed one hour later. The animals were sacrificed by cervical fracture and the carcinogen treated pouches were excised, fixed in 10% buffered formalin, and autoradiographs were prepared and stained by the method already described (Experiment 2).

The autoradiographs were examined at a magnification of 100 x 10 and the labelled cells in relation to 2000-3000 basal cells were counted in each animal and were expressed as a percentage of the number of basal cells. The position of the labelled cells was also noted as basal or suprabasal. Although occasional areas of focal epithelial atypia were observable, such/

such areas were intentionally excluded from the counts, and the percentages of labelled cells/basal cells represent that of regular epithelial hyperplasia without loss of stratification.

Part 2. Ten out of the 14 animals in this group were given intra-peritoneal injections of 4 mg. per kg. body weight of vinblastine sulphate in distilled water, while the other 4 animals served as controls, and were given comparable volumes of distilled water. Five of the experimental animals were sacrificed 2 hours after the injection of vinblastine, and the remaining 5 were sacrificed 6 hours after the injection. Two control animals were sacrificed along with each 5 experimental animals. Animals were sacrificed by cervical fracture and the treated pouches were excised, fixed in 10% buffered formalin, and paraffin sections 6 microns in thickness were prepared and stained with haematoxylin. The histological sections were examined at a magnification of 100 x 10 and the number of arrested metaphases in relation to 3000-4000 basal cells were counted in each animal in the experimental group, while the number of mitotic figures in relation to a similar number of basal cells were counted in each control animal. These counts were expressed as a percentage of the number of basal cells.

The mitotic rate was estimated by fitting a regression line through the origin for the data on arrested metaphases using the method of least squares.

Results:

The details of the percentage of labelled cells/basal cells is shown in Appendix 3 A.

The mean $\pm$ 1 standard error for Group 1 and Group 2 were found to be 11.01 $\pm$ 0.23%, and 12.99 $\pm$ 0.13% respectively. Hence the number of cells that entered the S-phase during the interval between injections, i.e.1.5hours, was (12.99 minus 11.01) 1.98 $\pm$ 0.27%. Therefore the rate of entry of cells into S-phase (rate of DNA synthesis) was 1.32 $\pm$ 0.18% per hour. The duration of S-phase was calculated using the formula:-

T_S/

Fig. 25.

Autoradiograph of hyperplastic cheek pouch epithelium of hamster sacrificed I hour after the injection of tritiated thymidine.

Haem. X100.

$$T_S = \frac{\text{Number of cells in S-phase}}{\text{Rate of entry into S-phase}}$$

$$= \frac{11.01}{1.32}$$

$$= 8.33 \text{ hours.}$$

From the above data an approximate estimate of the duration of the cell cycle was made by the application of Wright's hypothesis as follows:-

$$T_C = \frac{T_S \times N_C}{N_S}$$

$$= \frac{8.33 \times 100}{11.01}$$

$$= 75.7 \text{ hours.}$$

Out of the total of 4164 labelled cells counted in the experimental animals in both groups, 837 (20.1%) were present in the suprabasal layers. The presence of suprabasal labelled cells in hyperplastic epithelium is illustrated in Figure 25. On the assumption that all progenitor cells in the population proliferate at the same rate, 20% of the progenitor cells in the hyperplastic epithelium would be present in the suprabasal layers, and hence the above estimate of the duration of the cell cycle would prove to be lower than the actual duration. On the above assumption that 20% of the progenitor cells are present in the suprabasal layers, the estimate of the duration of the cell cycle would be $\frac{8.33 \times 120}{11.1}$, that is 90.8 hours.

Part 2.

The details of mitotic counts in the experimental and control animals is shown in Appendix 3 B. While Figure 26 illustrates the arrested metaphases in the hyperplastic epithelium of vinblastine injected animals, Figure 27 shows hyperplastic control epithelium.

The/

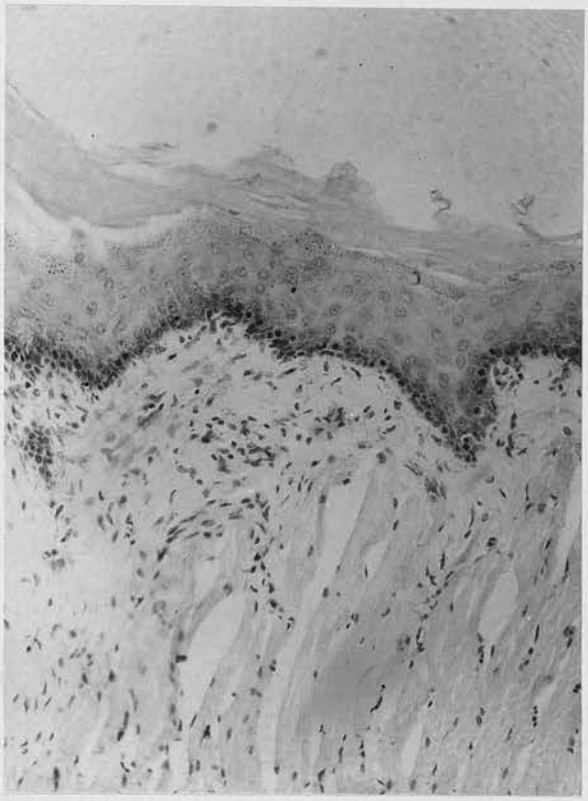

Fig. 26.

Hyperplastic epithelium of hamster sacrificed 6 hours after injection of vinblastine sulphate.

Haem. X40.

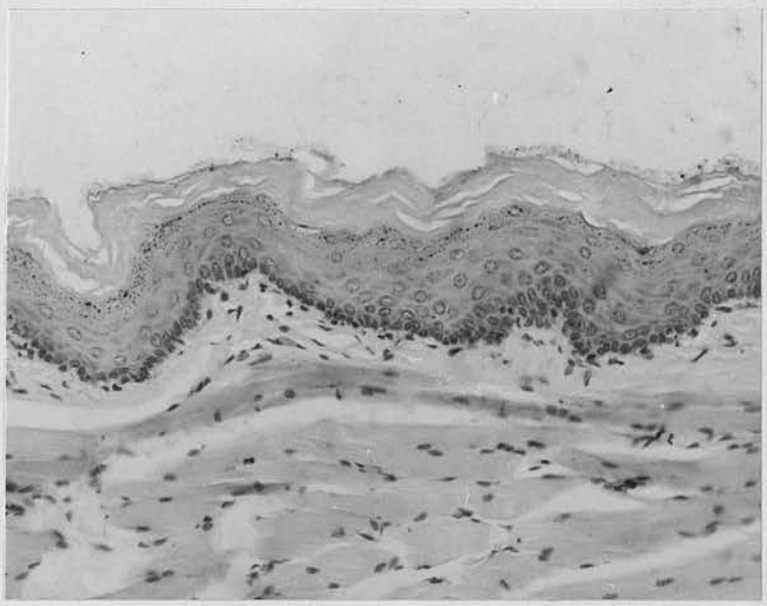

Fig. 27.

Hyperplastic cheek pouch epithelium.

Haem. X40.

The regression equation was found to be $y = 1.29\ x$, and the mitotic rate was estimated as $1.29 \pm 0.06\%$ per hour (Appendix 3 C). The percentage of mitosis/basal cells was found to be $2.04 \pm 0.2\%$. The mitotic duration was, therefore, estimated as follows:-

$$\text{Mitotic duration} = \frac{\text{Mitotic index}}{\text{Mitotic rate}}$$

$$= \frac{2.04}{1.29}$$

$$= 1.58 \text{ hours.}$$

The duration of $(T_{G1} + T_{G2})$ was estimated as T_C minus $(T_S + T_M)$, and was found to be 80.9 hours. T_{G1} and T_{G2} could not be estimated separately by the methods used in this study.

Discussion:

The number of cells in the S-phase between the first and the second injections of tritiated thymidine is proportional to the interval between the injections even if the injected thymidine was available only for a period less than one hour, which was the interval between the final injection and sacrifice in both groups. Assuming that N_S is the number of cells in S-phase at the time of injection, r is the rate of entry of cells into S-phase, t is the time for which tritiated thymidine was available to the cells (t is less than 1 hour), and LC_1 and LC_2 are the percentage of labelled cells in Group 1 and Group 2 respectively, then:-

$$LC_1 = N_S + r.t$$

$$LC_2 = N_S + 1.5\ r.t + r.t$$

$$\text{Therefore } (LC_2 - LC_1) = 1.5\ r.$$

This/

This relationship holds good provided that the interval between the injections is shorter than the duration of the S-phase so that no cell has entered and traversed through the S-phase during this interval, and hence failed to take up tritiated thymidine. In practice this interval is always small in comparison to the duration of S-phase, for the duration of the experiment is usually kept shorter than the suspected value of $T_{G2} + T_M$ for the particular cell population. This is necessary in order to minimise the errors due to the increase in the number of labelled cells as a result of some of these cells undergoing mitosis and doubling their numbers. It seems reasonable to assume that the error introduced by the doubling of labelled cells during the experimental period of 2.5 hours in the present study is negligible.

While studying the effects of carcinogen applications on the mitotic activity of mouse skin, Kiljunen (1956), and Evensen (1961) observed an increase in the mitotic indices of untreated areas of the skin in experimental animals. While this indicates the possible effect of the minute amounts of carcinogen that is absorbed through the skin, the observation of Dachi et al., (1967) that occasional tumours develop in the untreated pouches of animals painted unilaterally with 0.5% DMBA in dimethyl sulfoxide indicates the possibility of diffusion of the carcinogen into the contralateral cheek pouch. Hence it is doubtful whether the epithelium of the untreated cheek pouches of the experimental animals could be considered as representative of normal epithelium after long periods of carcinogen treatment. Therefore, comparisons of the cell cycle characteristics of the hyperplastic and neoplastic epithelia are made with those of the epithelium of unpainted control animals.

While the progenitor cells are confined to the basal cell layer of the normal cheek pouch epithelium, an additional sub-population of progenitor cells are present in the suprabasal layers of the hyperplastic epithelium. This constitutes about 20% of the population in the basallayer, and hence the estimates of/

of the indices and rates of mitosis and DNA synthesis based on 100 basal cells should be corrected by a factor of $\frac{100}{120}$. This becomes necessary for there are actually 120 progenitor cells in relation to 100 basal cells. The corrected indices and rates would then be given as:-

Mitotic index	=	1.70% of progenitor cells
Labelling index	=	9.17% of progenitor cells
Mitotic rate	=	1.08% progenitor cells per hour
Rate of DNA synthesis	=	1.10% progenitor cells per hour.

The mitotic and labelling indices of the hyperplastic epithelium are significantly higher than those of normal epithelium (Appendix 3 D).

The rate of transit of the cells through the cell cycle as estimated at the S-phase and at mitosis are close to each other. The rate of transit of the progenitor cells across the cell cycle, in the hyperplastic epithelium is about 79% higher than that in the normal cheek pouch epithelium (0.61% per hour).

Although the duration of S-phase and that of mitosis in epithelial hyperplasia is shorter than those of normal epithelium, the change is not inversely proportional to their respective rates and the reduction of the duration of the cell cycle appears to be effected mainly through a decrease in the duration of the G1-phase. The duration of $(T_{G1} + T_{G2})$ in hyperplastic epithelium is 80.9 hours, while that of normal epithelium is 151.9 hours. Although T_{G1} and T_{G2} have not been estimated separately in the hyperplastic epithelium the reduction in the value of $(T_{G1} + T_{G2})$ is mainly due to a shorter G1-phase irrespective of T_{G2}, especially in view of its short duration in comparison to that of the G1-phase.

The estimate of the duration of the cell cycle (90.8 hours) is approximate, for Wright's hypothesis is strictly applicable only to steady state systems. However, the cell kinetics of regular epithelial hyperplasia is closer to steady state growth than to true exponential growth. For conditions of/

of steady state to be satisfied, with every cell that is generated one cell should move out of each compartment so that the cell numbers remain constant. In exponential growth, however, the dividing cells double their numbers after an interval equal to the duration of the cell cycle so that if N_0 is the number of cells at a particular time, and T_C is duration of the cell cycle, then the number of N_t after t hours would be given as:-

$$N_t = N_0 \cdot 2^{t/T_C}$$

Although the number of progenitor cells as well as the total number of cells in the population have increased during the development of epithelial hyperplasia, the regular maturation and keratinisation of the epithelium indicates that there is migration of cells from the progenitor cell compartment to the maturing cell compartment. The development of epithelial hyperplasia is a result of the disparity between the rate of cell production, and the rate of cell loss from the epithelium. Hence it is reasonable to assume that the kinetics of regular epithelial hyperplasia is closer to steady state growth than to exponential growth. Departures from linear growth would only mean that the estimate of the duration of the cell cycle using Wright's hypothesis would be higher than the actual duration, and hence it could be stated that the duration of cell cycle of the progenitor cells of the hyperplastic epithelium is much shorter than that of normal epithelium.

Theoretically, epithelial hyperplasia could develop as a result of an increase in the rate of cell production or a decrease in the rate of cell loss with a consequent increase in the mean life span of the non-dividing cells in the population (delayed maturation) or both (Berenblum, 1957). The increase in the rate of cell proliferation could be brought about by:-

a) a decrease in the duration of the cell cycle and its phases with or without an increase in the number of progenitor cells

b/

b) an increase in the number of progenitor cells with or without a decrease in the duration of the cell cycle and its phases.

The results of the present study indicates that at the stage of regular epithelial hyperplasia there is: 1) an increase in the number of progenitor cells in the population, 2) an increase in the number of cells in DNA synthesis or mitosis at any one time, 3) a decrease in the duration of the cell cycle and its phases.

An increase in the number of progenitor cells (Van Scott & Ekel, 1963), and a decrease in the duration of the phases of the cell cycle (Weinstein & Frost, 1968) have been reported in relation to epithelial hyperplasia of psoriasis.

Although it was not investigated in the present study, it is also possible that the mean life span of the non-dividing cells in hyperplastic epithelium is greater than that of the normal counterpart.

Evensen (1962), and Skjaeggestad (1964), studying the mechanism of epithelial hyperplasia induced by carcinogenic agents, reported an initial increase in the rate of cell loss followed by an increased generation of cells. They considered that hyperplasia was the result of a 'population shift' caused by the injurious effects of the carcinogen. Skjaeggestad also stated that the kinetics of epithelial hyperplasia induced by carcinogens was not different from that caused by non-carcinogenic agents.

EXPERIMENT 5 A.

Studies on cell proliferation in DMBA induced squamous carcinomas in the hamster cheek pouch.

Introduction.

Tumours are considered to be examples of 'closed dividing' type of cell populations. An ideal closed dividing type of cell population would grow exponentially and double its cell numbers at intervals equal to the duration of the cell cycle, so that if there are N_0 cells in the population at a particular instance, the number of cells N_t after an interval t could be expressed as:

$$N_t = N_0 \cdot 2^{t/T_C} \quad \text{---------- (1)}$$

In exponentially growing populations the labelling and mitotic indices do not directly indicate the fraction of the cell cycle that is spent in DNA synthesis or mitosis (Baserga & Lisco, 1963), and hence Wright's hypothesis is no longer applicable.

Johnson (1960) has derived a relationship between the mitotic and labelling indices and their durations in exponentially growing cell populations. However, this relationship is based on the assumption that the duration of mitosis or DNA synthesis is short in comparison to that of the cell cycle. Although the assumption would hold good in the case of the former, the latter cannot always be assumed to be small in relation to the duration of the cell cycle. Hence the equation put forward by Johnson (1960) is more applicable to mitosis and is shown below:

$$\text{Fraction of cells in mitosis} = \log_e 2 \cdot \frac{T_M}{T_C} \quad \text{------ (2)}$$

Cleaver (1965), indicating the inapplicability of the above equation to the S-phase, put forward an equation to relate the labelling index with the duration of DNA synthesis, and the duration of the various phases of the cell cycle in exponentially growing cell populations. It is given as:

$$\text{Labelled fraction} = \exp \frac{(T_{G2} + \frac{1}{2}T_M)}{T_C} \cdot \ln 2 \left(\exp \frac{T_S}{T_C} \ln 2 - 1\right)$$

---------- (3)

The notations used for the duration of the cell cycle and its phases in the original equations of Johnson (1960), and Cleaver (1965) have been altered to those used in the present study.

While the above relationships are applicable to true exponential growth, in actual practice not all tumour cells are always capable of proliferation, and the ratio of the number of proliferating cells to the total number of cells in the population is referred to as the growth fraction (Mendelsohn, 1963). When all the cells in the population are capable of proliferation the growth fraction becomes one. The growth fraction in tumours is more often than not less than one.

While the rate of generation of tumour cells is a function of the growth fraction and the duration of the cell cycle, the rate of growth of tumours would depend on the balance between the rate of generation of cells and the rate of loss of cells from the tumour as a result of cell death, metastasis, desquamation, etc. Thus only in the ideal situation where there is no cell loss from tumours, they constitute true examples of the closed dividing type of cell populations.

The growth fraction of a cell population could be determined by a number of methods (Mendelsohn, 1963; Post & Hoffman, 1964a; Lala & Patt, 1966). However, the method based on the theoretical and observed labelling indices is the one of choice with an experimental design similar to that in the present study. The growth fraction could be expressed as the observed labelling index/theoretical labelling index (Post & Hoffman, 1964a; Lala & Patt, 1966). While the theoretical labelling index in a steady state system would be the ratio of the duration of S-phase to the duration of the cell cycle, this ratio does not express the number of cells in the S-phase of exponentially growing populations. The theoretical labelling index of such populations could, however, be determined using the equation put forward by Cleaver (1965) provided that the values of T_C and $(T_{G2} + \frac{1}{2} T_M)$ are known.

When/

When the growth fraction of a closed dividing type of cell population is less than one, then the cell numbers would increase by a factor α ($1 \leq \alpha \leq 2$ for expanding cell populations) instead of doubling their numbers at intervals equal to the duration of the cell cycle. If p represents the growth fraction or the proliferative fraction, then $p = (\alpha - 1)$ (Steel, 1967). The equation $N_t = N_0 \cdot 2^{t/T_C}$ (equation 1) could then be rewritten as:

$$N_t = N_0 \cdot (\alpha)^{t/T_C} \quad \text{---------- (4)}$$

When $N_t = 2 N_0$, the cell numbers in the population would have doubled and the interval after which this is achieved is referred to as the theoretical or potential doubling time of the population.

As already stated the size of tumours does not always increase in accordance with the predicted doubling time because of the presence of varying degrees of cell loss.

Object of the study.

The experiments to be described were planned to estimate the following parameters in chemically induced squamous cell carcinomas of the hamster cheek pouch:

1) the duration of the cell cycle and its phases,
2) the mitotic rate,
3) the growth fraction,
4) the theoretical doubling time.

Materials and Methods.

The right cheek pouches of 45 male hamsters of 2-3 months of age were painted with an 0.5% solution of DMBA in acetone, three times per week, for 12 weeks as already described (Experiment 3). While the majority of the animals in this experiment had a golden fur colour, there were also a few spotted (brown fur with white spots) and albino hamsters in this series (33 golden, 7 spotted, 5 albino). Although they belonged/

belonged to the fiftieth-sixtieth generation of line-breeding, originally starting from 3 animals, the albino and spotted hamsters have been bred separately for a number of generations.

The carcinogen treated pouches were examined before each painting for the appearance of tumours, and the time taken for the appearance of the first tumour in each animal was noted. Although some of the animals developed more than one tumour, only the time taken for the appearance of the first tumour in each animal was used in the determination of the mean latent period for the development of tumours.

Ten painted animals died during the experimental period and all the remaining animals had developed tumours by the thirteenth week from the beginning of painting as by gross appearances. The experiments to be described were performed after a further period of two weeks. The animals were divided at random into two groups, one of 27 animals and the other 8 animals.

Part 1.

Twenty-seven experimental animals were given intra-peritoneal injections of 1 micro-curie per gm. body weight of tritiated thymidine (specific activity 5 curies/milli-mole), all the animals being injected within a period of 20 minutes. The first animal was sacrificed 30 minutes after the injection of tritiated thymidine, and thereafter one animal every hour up to 26 hours.

The animals were sacrificed by cervical fracture and the carcinogen treated pouches were excised and fixed in 10% buffered formalin. After adequate fixation the tissues were trimmed and paraffin sections 6 microns in thickness were prepared at an interval of 30 microns from each other. Autoradiographs were prepared and stained as described earlier (Experiment 2).

The autoradiographs were examined at a magnification of 100 x 10 and scores of labelled and unlabelled mitoses in relation to squamous cell carcinomas were made in each autoradiograph. The percentage of labelled mitoses/total mitoses was computed by aggregating/

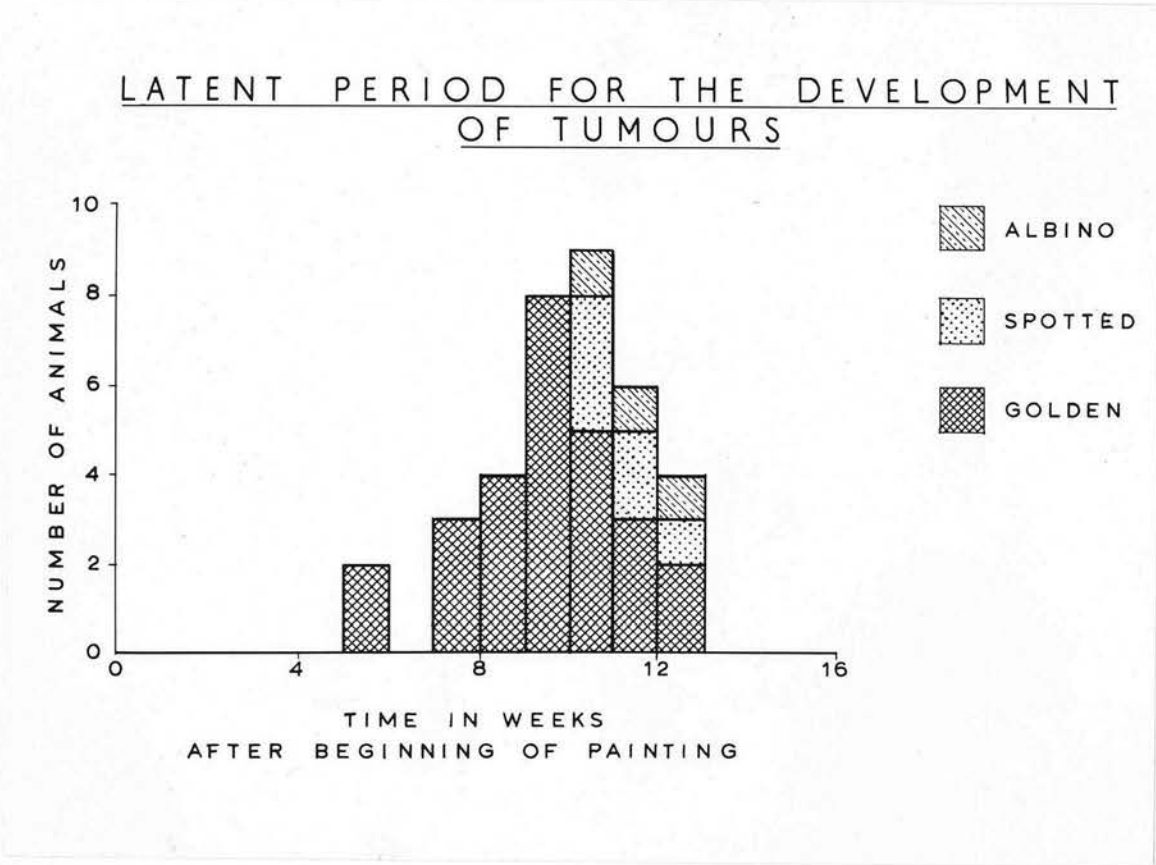
LATENT PERIOD FOR THE DEVELOPMENT OF TUMOURS
NUMBER OF ANIMALS
0
2
4
6
8
10
0
4
8
12
16
TIME IN WEEKS
AFTER BEGINNING OF PAINTING
ALBINO
SPOTTED
GOLDEN

Fig. 28.

aggregating the scores from 5 autoradiographs in each animal and in most cases this aggregate was greater than 100. Plots of percentage labelled mitoses/total mitoses versus time were made.

The labelling and mitotic indices were determined by counting about 2000 cells in each tumour in animals sacrificed up to 5 hours from the time of injection of tritiated thymidine. The areas counted within each tumour was selected at random. The tumours in animals sacrificed after 5 hours were not included in order to minimise errors due to increments in the number of labelled cells as a result of mitosis.

Part 2.

Eight animals were given intra-peritoneal injections of 4 mg. per kg. body weight of vinblastine sulphate in distilled water. Four of the animals were sacrificed 2 hours after the injection, and the other 4 were sacrificed 5 hours after injection. The tissue sections were prepared in the same manner as described in Part 1 of the experiment, and were stained with haematoxylin and eosin. The sections were examined at a magnification of 100 x 10 and the number of arrested metaphases in relation to 2000 cells were counted in each tumour and were expressed as a percentage of the number of tumour cells.

The mitotic rate was estimated by fitting a regression line through the origin for the data on arrested metaphases by the method of least squares.

Results.

The gross changes observed during the period of carcinogen treatment was similar to those described previously (Experiment 3). The latent period for the development of tumours with reference to fur colour of the animals is shown in Figure 28. The mean ± 1 standard error of latent period for the development of tumours in golden hamsters was 9.34± 0.4 weeks, while that in spotted and albino hamsters were 10.6± 0.3 weeks and 11± 0.6 weeks, respectively.

Since/

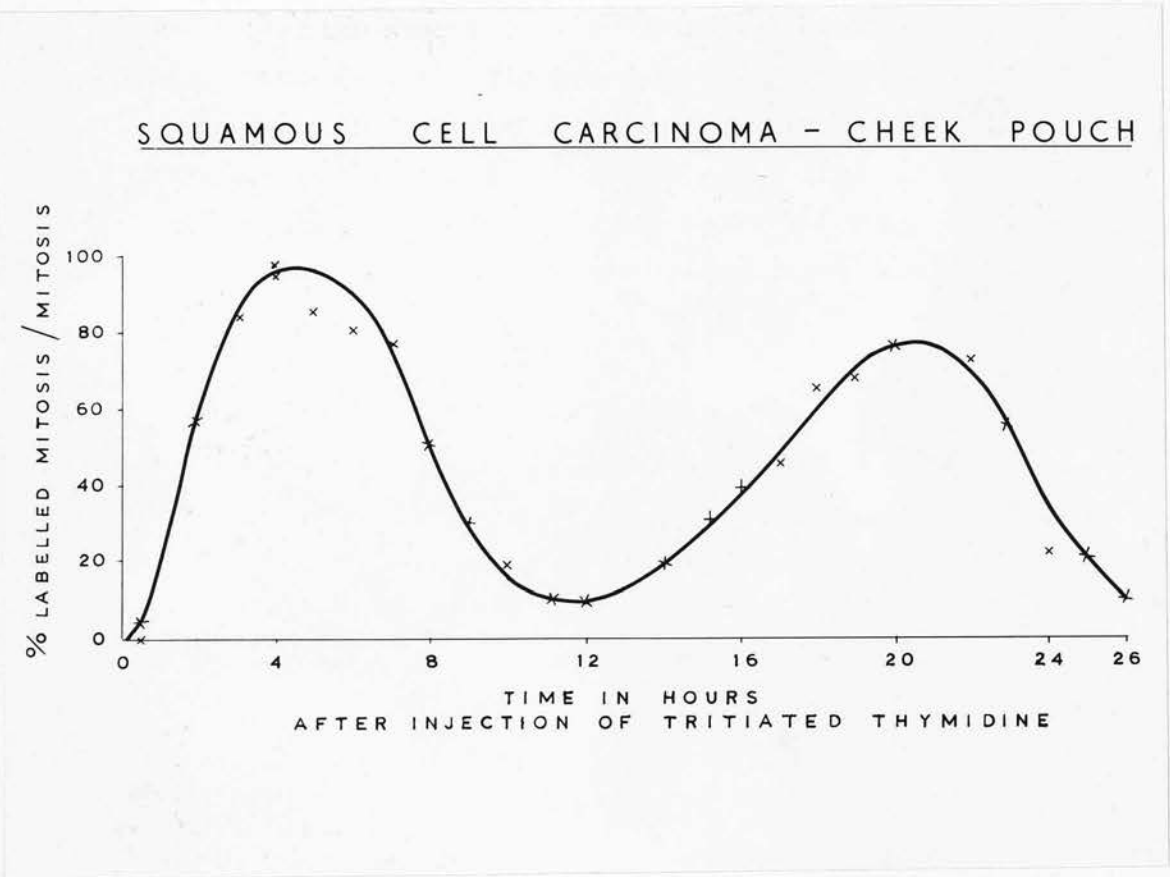
SQUAMOUS CELL CARCINOMA - CHEEK POUCH
% LABELLED MITOSIS / MITOSIS
100
80
60
40
20
0
0
4
8
12
16
20
24
26
TIME IN HOURS
AFTER INJECTION OF TRITIATED THYMIDINE

Fig. 29.

Since the albino and spotted groups were small and the mean latent period for development of tumours is about the same in these groups, it was thought desirable to pool the data for these groups before testing for significant differences between golden hamsters and albino and spotted hamsters. The latent period for the development of tumours in golden hamsters was found to be significantly shorter than that for the rest of the animals (Appendix 4A).

On histological examination of the autoradiographs and the stained sections obtained from each animal, it was found that only 30 out of the 35 pouches that were excised had developed squamous cell carcinomas. The other 5 (3 in Part 1 and 2 in Part 2 of the experiment) showed pappilomas and areas of carcinoma in situ, and were excluded from the study. The details of the percentage of labelled mitoses/total mitoses are given in Appendix 4B.

The plots of percentage of labelled mitoses/total mitoses versus time after injection of tritiated thymidine for the tumours is shown in Figure 29. The first wave of percentage labelled mitosis rises steeply to reach 98%, four hours after injection and then to fall to about 7% between 11 and 12 hours after injection. The second wave of labelled mitoses gradually rises from this point, but with a lesser gradient than that for the ascending limb of the first wave. It reaches a peak at about 80%, twenty hours after injection, and then falls gradually.

The estimates of the duration of the cell cycle and its phases obtained through the 50% intercepts on the waves of labelled mitoses are as follows:

T_S = 6.2 hours

$(T_{G2} + \frac{1}{2}T_M)$ = 1.8 hours

T_C = 15.4 hours (distance between 50% intercepts on ascending limbs).

The/

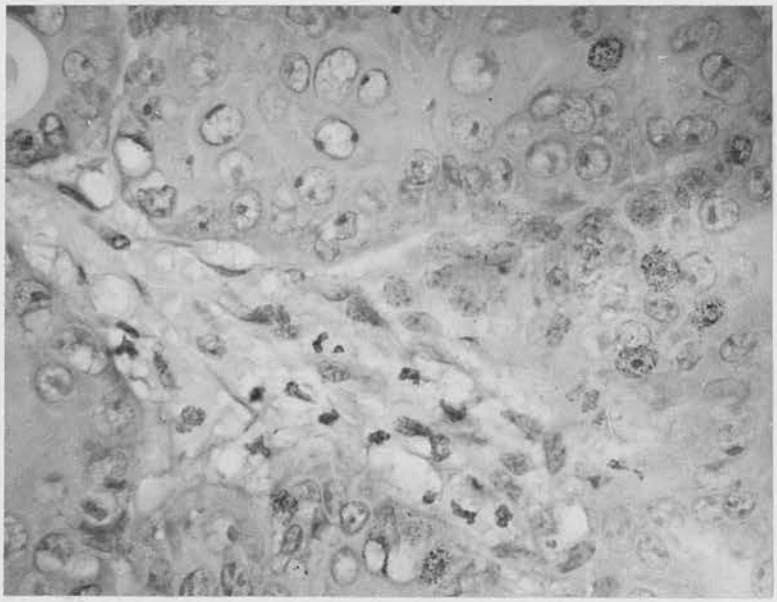

Fig. 30.

Autoradiograph of squamous cell carcinoma in pouch of hamster sacrificed 30 minutes after the injection of tritiated thymidine.

Haem. X100.

The distance between the 50% intercepts on the descending limbs was found to be 15. 2 hours, while the distance between the first and second peaks of labelled mitoses was found to be 16 hours. Thus the average value of the duration of the cell cycle from these three measurements would be 15.5 hours.

The details of labelling and mitotic indices are shown in Appendix 4B. The pattern of labelling of tumour cells is illustrated in Figure 30. The mean $\pm$ 1 standard error of labelling and mitotic indices were found to be 21.7 $\pm$ 0.45% and 2.04 $\pm$ 0.08% respectively.

The growth fraction of the tumour cell population was determined by the method of theoretical and observed labelling indices. The theoretical labelling index was determined using equation (3) (page 107). Thus:

$$\text{Labelled fraction} = \exp \frac{(T_{G2} + \frac{1}{2} T_M)}{T_C} \ln 2 \left(\exp \frac{T_S}{T_C} \ln 2 - 1\right)$$

$$= \exp \frac{1.8}{15.5} \times 0.693 \left(\exp \frac{6.2}{15.5} \times 0.693 - 1\right)$$

$$= 0.3488$$

$$\text{Therefore, growth fraction} = \frac{\text{Observed labelling index}}{\text{Theoretical labelling index}}$$

$$= \frac{21.7}{34.88}$$

$$= 0.62$$

Thus only 62 out of 100 cells of the population are proliferating and therefore the number of cells in mitosis for every 100 proliferating cells would become $2.04 \times \frac{100}{62}$ %, that is, 3.29%, because the estimate of 2.04% was based on the total cell population. The mitotic duration could then be estimated using the equation:

$$\text{Fraction of cells in mitosis} = \log_e 2 \frac{T_M}{T_C}$$

$$T_M = \frac{0.0329 \times 15.5}{0.693}$$

$$= 0.74 \text{ hours.}$$

Thus/

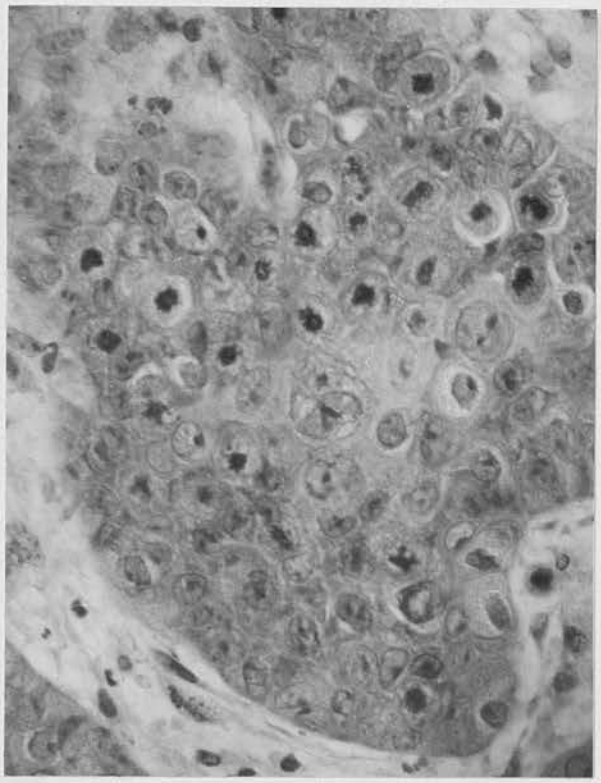

Fig. 31.

Squamous cell carcinoma in cheek pouch of hamster sacrificed 5 hours after injection of vinblastine sulphate.

H. & E. X100.

$$\text{Thus, } T_{G2} = (T_{G2} + \tfrac{1}{2} T_M) - \tfrac{1}{2} T_M$$

$$= (1.80 - 0.37) \text{ hours}$$

$$= 1.43 \text{ hours.}$$

$$\text{Therefore, } T_{G1} = T_C - (T_S + T_{G2} + T_M)$$

$$= 15.5 - (6.2 + 1.43 + 0.74)$$

$$= 7.13 \text{ hours.}$$

Part 2.

The metaphase arrest produced by vinblastine in squamous cell carcinoma of the hamster cheek pouch is illustrated in Figure 31. The details of the percentage of metaphases/tumour cells in the two groups is shown in Appendix 4C. The regression equation was found to be $y = 2.62\ x$ and the mitotic rate was estimated as $2.62 \pm 0.09\%$ of the population per hour (Appendix 4D). Therefore the rate at which the proliferating cells are dividing would be $2.62 \times \frac{100}{62}$ %, that is 4.23% of the proliferating cells in the population per hour. Estimate of the mitotic duration could then be made as:

$$\text{Mitotic duration} = \frac{\text{Mitotic index}}{\text{Mitotic rate}}$$

$$= \frac{3.29}{4.23}$$

$$= 0.78 \text{ hours.}$$

Discussion.

Although the study was not planned to investigate the differences in the susceptibility of different strains of hamsters to experimental carcinogenesis, the latent period for the induction of tumours in golden hamsters was found to be shorter than the albino and spotted hamsters under the conditions of the present study. Reiskin & Berry (1968), however/

however, reported that albino hamsters were more susceptible to carcinogenesis than golden hamsters. Although the very small number of albino hamsters in this study do not allow a firm conclusion to be drawn, it is interesting to note that the latent period for development of tumours in golden hamsters in the present study is shorter than that reported by Reiskin & Berry (1968). While the latent period for induction of tumours in golden hamsters when painted with 0.5% DMBA in acetone was 9.34 ± 0.3 weeks in the present study, Reiskin & Berry (1968) reported a latent period of 10.7 ± 0.44 weeks in spite of the fact that in the latter study the carcinogen DMBA was dissolved in mineral oil, a solvent that enhances carcinogenesis (page 21). This finding strongly suggests that the susceptibility to carcinogenesis depends on the genetic background of the experimental animals. These findings support those of Morris (1961) who reported that animals of different genetic backgrounds had different susceptibility to experimental carcinogenesis. It also appears that the susceptibility to carcinogenesis cannot always be determined by the fur colour of the experimental animals.

The duration of the cell cycle of the tumour cells was found to be about a tenth of that of progenitor cells of the normal pouch epithelium. However, this alteration in the duration has been brought about by dissimilar alterations in the duration of the various phases of the cell cycle. While the duration of S-phase is reduced by about 40%, the duration of mitosis in tumour cells is less than half of that in normal cells and the duration of G_1-phase is only one-twentieth of that of normal cells. The duration of G2-phase, however, has only been reduced from 1.92 hours to 1.43 hours.

Although the results of the present study and that of Reiskin (1968) have shown that the duration of the cell cycle of chemically induced tumours in the hamster cheek pouch is shorter than that of the normal epithelium and that the rate of cell proliferation in tumours is higher than that in the normal/

normal counterpart. Evensen & Iversen (1962) and Post & Hoffman (1964b) have shown that there is an increase in the duration of the cell cycle as well as a decrease in the rate of cell proliferation of cells in induced tumours. These findings support the view that tumour growth is essentially a result of abnormal cell proliferation rather than a rapid cell proliferation, although rapid cell proliferation could contribute towards abnormal cell proliferation. Since the rate of growth of a cell population depends on the balance between the rate of cell production and the rate of loss of cells from the population, a disturbance of the tissue homeostasis leading to delayed maturation of cells could lead to progressive growth of the cell population. As Baserga (1965) has pointed out, a few normal cell populations proliferate at a greater rate than many tumours. While these normal cell populations proliferate in an orderly fashion leading to regular maturation and loss of cells, there is no co-ordination between cell production and cell loss in tumour cell populations.

The growth fraction of the tumours was found to be 0.62. Thus only 62% of the tumour cells were proliferating. The theoretical or potential doubling time could then be estimated using equation (5) (Appendix 4 E):

$$t = T_C \times \frac{\log_e 2}{\log_e (1+p)}$$

$$= 15.5 \times \frac{\log_e 2}{\log_e 1.62}$$

$$= 15.5 \times \frac{0.6931}{0.4824}$$

$$= 22.3 \text{ hours.}$$

Reiskin & Berry (1968) estimated the doubling time of DMBA induced tumours in the cheek pouch of golden hamsters as 12.3 days by measurements of tumour size. While the estimate of the theoretical/

theoretical doubling time does not take into account the loss of cells from tumours, the estimate of the doubling time by measurements of tumour size could be influenced by the stroma, direction of growth of the tumours, etc. Reiskin & Berry have estimated the growth fraction of squamous cell carcinomas of the cheek pouch of golden hamsters as 0.04. Their growth fraction was estimated through equation (5) using the observed doubling time for the value of t. The observed doubling time of tumours depends not only on the growth fraction and the duration of the cell cycle, but also on the rate at which cells are being lost from the tumours. The observed doubling time, therefore, is bound to be greater than the theoretical doubling time and hence the growth fraction estimated by the use of the observed doubling time would not represent the fraction of the tumour cells that are proliferating.

It appears that the growth fraction could vary with time. Lala & Patt (1966), studying the growth fraction and the duration of the cell cycle of mouse ascites tumours at various intervals after inoculation, found that there was progressive decrease in the growth fraction from 0.82 on the first day to 0.53 by the seventh day. This was accompanied by an increase in the duration of the cell cycle from 17 hours to 22 hours. A similar inverse relationship between growth fraction and the duration of the cell cycle has also been reported in the normal hepatocytes of young rats (Post & Hoffman, 1964a). The growth fraction of solid tumours, however, does not seem to change considerably within an interval of one week (Mendelsohn, 1965).

Varying terminology has been used for the non-proliferating tumour cells. Steel (1967), following the nomenclature of Cairne et al. (1965), has called them Q-cells in distinction to the P-cells which are the proliferating cells of the population. Reiskin & Berry (1968) suggested that any 'proliferatively capable non-dividing cell' should be called a T_0 cell in preference to a G_0 cell, for it is not known whether the tumour cells are resting in the G1 or G2 phases. Mendelsohn & Dethlefsen (1969) divided the non-proliferating tumour cells G_0 cells that are capable of either reverting into proliferation or progressing into cells that are permanently incapable of proliferative activity, and sterile cells which cannot revert to proliferative activity.

The/

The existence of proliferatively capable cells in tumours would not only prove hazardous, for they form a fraction that would prove resistant to chemotherapeutic, as well as radiotherapeutic, measures ultimately capable of proliferation after long intervals of time, leading to reappearance of the growth, but they are also capable of affecting rates of tumour growth by movement into or out of the proliferating compartment even in the absence of any alterations in the duration of the cell cycle of the tumour cells.

EXPERIMENT 5 B.

Cell cycle studies on DMBA induced pre-neoplastic hyperplasia of the hamster cheek pouch.

Introduction.

The epithelium surrounding the tumours (Experiment 5A) was found to be hyperplastic and hyperkeratotic. The degree of hyperplasia and hyperkeratosis was more marked than those observed in regular epithelial hyperplasia (Experiment 4). There was a great variation in the thickness of the epithelium along its length with varying degrees of loss of stratification, and areas of carcinoma *in situ*. Thus the cell population surrounding the tumours is heterogenous. In view of this heterogenity the areas studied were confined to areas of epithelial hyperplasia without marked loss of stratification. The term pre-neoplastic hyperplasia is used to distinguish the hyperplastic epithelium surrounding the tumours from regular epithelial hyperplasia that develops after 5 weeks of carcinogen treatment (Experiment 4).

Materials and Methods.

The material used in this study consisted of the autoradiographs prepared in experiment 5A. The autoradiographs were examined at a magnification of 100 x 10 and the number of labelled and unlabelled mitoses in relation to areas of epithelial hyperplasia were scored. The percentage of labelled mitoses/total mitoses was computed by aggregating the scores from 5 autoradiographs and plots of percentage labelled mitoses/mitoses against time were made. The percentage of suprabasal labelled cells was estimated by counting 2500 labelled cells in autoradiographs obtained from animals sacrificed up to 5 hours after the injection of tritiated thymidine. The number of mitotic figures in relation to these labelled cells was also scored.

Results/

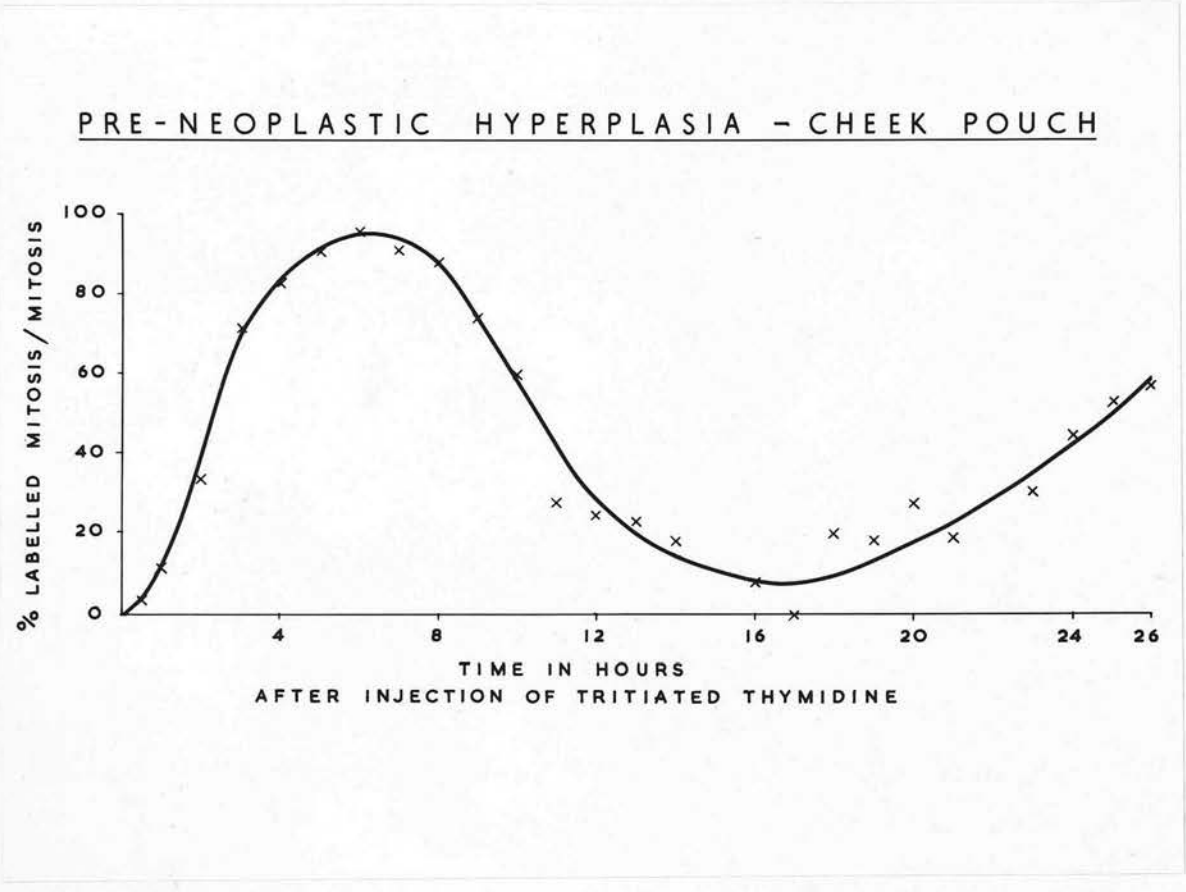
PRE-NEOPLASTIC HYPERPLASIA - CHEEK POUCH
% LABELLED MITOSIS / MITOSIS
100
80
60
40
20
0
4
8
12
16
20
24
26
TIME IN HOURS
AFTER INJECTION OF TRITIATED THYMIDINE

Fig. 32.

Results.

The details of the percentage labelled mitoses/total mitoses are shown in Appendix 5. The plots of percentage labelled mitoses/total mitoses against time is shown in Figure 32. The first wave of labelled mitoses increases gradually to reach 95%, six hours after injection of tritiated thymidine, then to fall with a lesser gradient. The wave reaches its minimum of about 8% at 16 hours and then to rise gradually. The gradient of the ascending limb of the second wave of labelled mitoses is lesser than that of the ascending limb of the first wave and the points show a greater scatter. The estimates of the duration of the phases of the cell cycle as made through the 50% intercepts on the ascending limbs of the first and second waves of labelled mitoses are as follows:

$$T_S = 8.2 \text{ hours}$$

$$(T_{G2} + \tfrac{1}{2} T_M) = 2.2 \text{ hours}$$

$$T_C = 22.7 \text{ hours.}$$

There were 269 mitotic figures in relation to the 2500 labelled cells that were scored in animals sacrificed within the first 5 hours. Since the number of progenitor cells in relation to the mitotic figures and labelled cells is the same, an approximate estimate of the duration of mitosis could be made as follows:

$$\frac{269}{2500} = \frac{T_M}{T_S}$$

$$T_M = 8.2 \times \frac{269}{2500}$$

$$= 0.88 \text{ hours.}$$

Therefore, $T_{G2} = (T_{G2} + \tfrac{1}{2} T_M) - \tfrac{1}{2} T_M$

$$= (2.2 - 0.44)$$

$$= 1.76 \text{ hours.}$$

T/

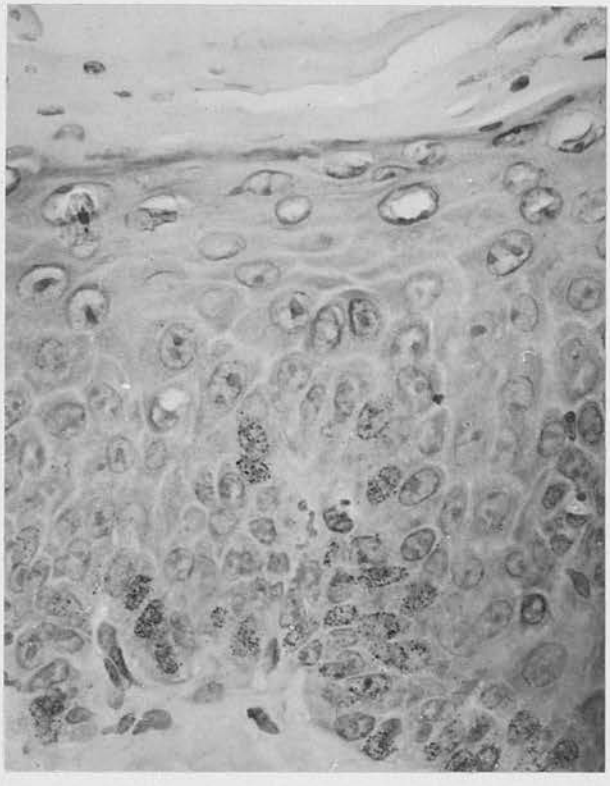

Fig. 33.

Autoradiograph of pre-neoplastic cheek pouch epithelium of hamster sacrificed 30 minutes after the injection of tritiated thymidine.

Haem. X100.

$$T_{G1} = T_C - (T_S + T_{G2} + T_M)$$

$$= 22.7 - (8.2 + 1.76 + 0.88)$$

$$= 11.93 \text{ hours.}$$

Out of the 2500 labelled cells that were scored, 1090 (43.6%) were found to be present in the suprabasal layers. The presence of labelled cells in the suprabasal layers of the hyperplastic epithelium is illustrated in Figure 33.

Discussion.

The duration of the cell cycle of the proliferating cells of pre-neoplastic hyperplasia was found to be 22.7 hours, which is shorter than the duration of the cell cycle in regular epithelial hyperplasia (90.8 hours), but longer than that of squamous cell carcinomas. The pattern of the reduction of the duration of the cell cycle in pre-neoplastic hyperplasia is similar to that described for regular hyperplasia and squamous cell carcinomas.

The mitotic and labelling indices were not estimated because of the wide variation in the thickness and the number of cells in the different parts of the epithelium. Such variations would make these estimates inaccurate because the mitotic figures and labelled cells are not uniformly distributed within the cell population.

The estimate of the duration of mitosis is approximate for the computation assumes that the duration of S-phase is short in relation to the duration of the cell cycle. Hence the estimate of the duration of G2-phase will be subjected to the accuracy of the estimate of the mitotic duration and can only be considered approximate.

Reiskin & Berry (1968) estimated the duration of cell cycle in pre-neoplastic epithelium of dark eared albino hamsters as 61.1 hours. Their estimates were based on calculations using the duration of S-phase and the labelling index which was the number of labelled cells expressed as the percentage of the total number of cells in the population. As suspected by Reiskin & Berry/

Berry (1968) this is a gross overestimate of the duration of the cell cycle. Direct measurements of the duration of the cell cycle from the waves of percentage labelled mitosis in this study has proved it. The duration of S-phase in the hyperplastic epithelium of dark eared albino hamsters was found to be 10.38 hours (Reiskin & Berry, 1968) in comparison to the duration of the normal cheek pouch epithelium of albino hamsters which was 9.3 hours. While the duration of S-phase in pre-neoplastic hyperplasia (8.2 hours) was found to be shorter than that of normal cheek pouch epithelium (10 hours) in the present study, Reiskin & Berry (1968) observed that the duration of S-phase in pre-neoplastic hyperplasia was greater than that of the corresponding normal cheek pouch epithelium. This may partly be due to the difference in the methods used in the estimation of the duration of S-phase.

While the duration of S-phase was estimated as the distance between the 50% intercepts on the ascending limbs of the first and second waves of labelled mitosis in the present study, Reiskin & Berry (1968) estimated it as the area under the first wave of labelled mitosis. Takahashi (1966), investigating the theoretical basis of the labelled mitosis curve, stated that the best method of estimating the duration of the cell cycle and its phases was through the 50% intercepts, provided the peak reached by the waves of labelled mitoses was greater than 60%. Thus the estimate of the duration of S-phase in the pre-neoplastic epithelium of albino hamsters may be higher than the actual value, especially in view of the scatter of the individual points that was observed by Reiskin & Berry (1968).

About 44% of the labelled cells were found to be present in the suprabasal layers of the hyperplastic epithelium in the present study. While it could be concluded that 44% of the progenitor cells are present in the suprabasal layers of the epithelium in pre-neoplastic hyperplasia, the general applicability of this value has its reservations. Since the nature/

nature of the cell population that surrounds the tumours is heterogenous, the above findings are only strictly applicable to the type of cell population defined earlier. Under the conditions of the study the percentage of the suprabasal progenitor cells in pre-neoplastic hyperplasia was found to be more than twice that in regular epithelial hyperplasia (20%).

General Discussion and Conclusions.

The stratified squamous epithelia of the adult are typical examples of steady state renewal systems. The rate and location of mitosis, and the duration of the cell cycle and its phases, are different in the various steady state systems as seen in those of the cheek pouch and the palate (Experiment 2). The only common feature shared by steady state systems is that the rate of cell production in these populations is in balance with their own rates of cell loss.

While the progenitor cells were confined to the basal cell layer of the cheek pouch epithelium, mitotic figures and labelled cells were also observed in the suprabasal layers of the epithelium of the palate. While it seems certain that about one-third of the progenitor cells in the palatal epithelium were present in the suprabasal layers, the nature of origin of these cells is uncertain, but has two possibilities. One is that these are stem type of cells that are inherently present in the suprabasal layers (page 44), and the other is that these are of the dividing transit type of cells originating from the basal cells, i.e. a proportion of the cells leaving the basal layer are capable of participating in the cell cycle. The latter suggestion does not compromise with the possibility that these are mitotic figures and labelled cells of the basal layer that are being pushed out of it as a result of population pressure.

While about 20% of the labelled cells were present in the suprabasal layers of carcinogen induced regular epithelial hyperplasia (Experiment 4), this percentage had increased to about 44% in pre-neoplastic hyperplasia (Experiment 5B) of hamster cheek pouch. While cells that migrate out of the basal layer of the normal cheek pouch epithelium undergo regular maturation/

maturation and keratinisation, during carcinogenesis a proportion of the cells leaving the basal layer retain the ability to undergo cell division. This proportion gradually increases as evidenced by the presence of twice as many labelled cells in the suprabasal layers of pre-neoplastic hyperplasia as there are in the suprabasal layers of early regular epithelial hyperplasia, ultimately leading to a loss of stratification so that the dividing and non-dividing cells become mixed with each other.

There is a progressive increase in the number of cells in DNA synthesis and mitosis, together with an increase in the rate at which these cells progress through the cell cycle. This is accompanied by a progressive decrease in the duration of the cell cycle. The duration of the cell cycle and its phases at the various stages studied during carcinogenesis of the hamster cheek pouch is shown below:

	T_C hrs.	T_S hrs.	T_{G2} hrs.	T_{G1} hrs.	T_M hrs.
Normal cheek pouch	163.9	10.0	1.92	150	1.77
Regular epithelial hyperplasia	90.8	8.33		80.9	1.58
Pre-neoplastic hyperplasia	22.7	8.2	1.76	11.93	0.88
Squamous cell carcinoma	15.4	6.2	1.43	7.13	0.74

While the above estimates for the duration of the phases of the cell cycle at different stages during carcinogenesis have shown that all the phases of the cell cycle are variable, the degree of reduction of the duration of each phase achieved at the different stages during carcinogenesis is not similar. The drastic reduction in the duration of the cell cycle from 163.9 hrs. in the normal cheek pouch to 15.4 hrs. is essentially due to shortening/

shortening of the duration of the G_1 - phase from about 150 hours to 7.13 hours. In comparison to this, the reduction in the duration of the other three phases of the cell cycle during development of squamous cell carcinomas amounts only to 5.32 hours. Thus the earlier suggestion that the G_1-phase is the one of most variable duration remains tenable.

It was found that vinblastine sulphate in a dose of 4 mg. per kg. body weight of the animal produced effective arrest of mitosis at the metaphase stage (Experiment 1). The estimates of the rate and duration of mitosis by the method of metaphase arrest by vinblastine (Experiments 4 & 5A) were close to the values obtained by tritiated thymidine autoradiography. However, the latter method allows much more valuable information to be gathered.

The growth fraction of squamous cell carcinomas was found to be 0.62. Considering that the normal cheek pouch epithelium consists of 3 - 4 cell layers and that only the basal cells are capable of proliferation, then the ratio of the proliferating cells to the total number of cells (growth fraction) would be about 0.25 - 0.35. Thus the fraction of proliferating cells in tumours is greater than that of normal epithelium.

The appearance of progenitor cells in the suprabasal layers of epithelial hyperplasia prior to development of tumours and the increased number of proliferating cells per unit cell mass of the tumours indicate that there is delayed maturation and keratinisation of the population leading to more cells participating in the cell cycle. Bullough (1965) suggests that delayed maturation and the progressive increase in the rate of generation of cells is possibly due to a progressive breakdown of the organisation of the tissue due to the gradual removal of the restraint on mitotic activity that is present in normal tissues.

Abbreviations Used in the Tables.

A-T	-	Ana- telophases
BM	-	Basal Mitosis
C	-	Cells
LC	-	Labelled Cells
LM	-	Labelled Mitosis
LMB	-	Labelled Mitosis, Basal
LSBM	-	Labelled Suprabasal Mitosis
M	-	Mitosis
Meta-	-	Metaphase
Pro-	-	Prophase
S. basal	-	Supra Basal
SBM	-	Suprabasal Mitosis
S.Error	-	Standard Error

APPENDIX 1 A

The number of cells observed in the different phases of mitosis expressed as a percentage of the number of basal cells.

(A) Cheek Pouch.

	Group 1			Group 2			Group 3			Group 4		
Time After Injection	Pro-	Meta-	A - T	Pro-	Meta-	A - T	Pro-	Meta-	A - T	Pro-	Meta-	A - T
½ hour	0.92	1.19	0.24	0.75 0.77	0.84 1.28	0.07 0.11				0.80 0.86	0.90 1.36	0.14 0.06
1 hour				0.94 0.82	1.25 1.48	0.23 0.17	0.96 0.90	2.04 2.28	0.00 0.16	0.91 0.81	1.30 1.36	0.00 0.00
2 hours	0.71	0.87	0.44	0.95 0.93	1.78 1.49	0.23 0.11	0.82 0.80	2.74 2.71	0.01 0.08			
4 hours	0.84	0.82	0.24	0.92 0.85	1.26 2.00	0.20 0.06	0.98 0.94	4.78 4.35	0.02 0.01	0.81 0.86	5.11 6.48	0.00 0.00
8 hours	0.84			0.84	1.64	0.09	0.84	4.08	0.00	0.74 0.64	6.14 8.36	0.00 0.00

APPENDIX 1 A

(b) Hard Palate.

Time After Injection	Group 1			Group 2			Group 3			Group 4		
	Pro-	Meta-	A - T	Pro-	Meta-	A - T	Pro-	Meta-	A - T	Pro-	Meta-	A - T
½ hour	1.19	1.97	0.29	1.22 1.19	2.57 3.91	0.18 0.00				1.48 1.72	3.41 3.76	0.04 0.02
1 hour				1.32 1.95	4.26 5.24	0.05 0.05	1.27 1.67	5.00 6.05	0.00 0.00	1.28 1.42	4.88 5.71	0.00 0.00
2 hours	1.59	1.88	0.54	1.58 1.83	7.57 6.67	0.19 0.29	1.66 1.29	9.14 9.25	0.08 0.01			
4 hours	1.72	1.60	0.58	1.75 1.42	9.35 9.73	0.38 0.05	1.66 1.46	17.93 11.71	0.00 0.02	1.44 1.65	14.64 16.22	0.00 0.00
8 hours	1.22	1.08	0.28	1.80	16.22	0.07	1.89	20.02	0.07	1.32 1.56	23.92 19.60	0.00 0.00

APPENDIX 1 A

(C) Ventral Surface of Tongue.

	Group 1			Group 2			Group 3			Group 4		
Time After Injection	Pro-	Meta-	A - T	Pro-	Meta-	A - T	Pro-	Meta-	A - T	Pro-	Meta-	A - T
½ hour	1.59	2.87	1.35	1.80 1.56	3.36 4.32	1.24 0.82				1.95 1.59	2.67 4.41	0.75 0.05
1 hour				1.37 1.77	5.07 5.23	0.44 0.69	2.10 1.55	4.84 6.29	0.39 0.09	1.91 2.63	5.55 7.76	0.06 0.00
2 hours	1.37	2.29	1.64	1.46 1.97	3.59 6.85	0.10 1.31	2.30 1.57	11.82 9.38	0.34 0.28			
4 hours	2.03	1.89	1.06	2.03 1.90	4.84 4.44	0.76 0.60	1.49 2.21	14.61 16.68	0.12 0.20	1.71 2.19	17.97 15.98	0.00 0.00
8 hours	1.53	1.24	0.92	1.78 1.66	 5.97	 0.30	1.96	11.73	0.13	1.62 1.81	20.80 21.45	0.00 0.00

APPENDIX 1 B.

When $y = b \cdot x$

$$b \text{ (Slope)} = \frac{\Sigma xy}{\Sigma x^2}$$

$$S^2 = \frac{\Sigma y^2 - \frac{(\Sigma xy)^2}{\Sigma x^2}}{(n-1)}$$

$$= \frac{\Sigma y^2 - b \cdot \Sigma xy}{(n-1)}$$

$$\text{Variance of (b)} = \frac{S^2}{\Sigma x^2}$$

$$\text{Standard error of (b)} = \sqrt{\frac{S^2}{\Sigma x^2}}$$

APPENDIX 1 C

% Prophases/Basal Cells - Pouch.

Time After Injection	Group 1	Group 2	Group 3	Group 4
½ hour	0.92	0.75 0.77		0.80 0.86
1 hour		0.94 0.82	0.96 0.90	0.91 0.81
2 hours	0.71	0.95 0.93	0.82	
4 hours	0.84	0.92 0.85	0.98 0.94	0.81 0.86
8 hours	0.84	0.84	0.84	0.74 0.64

Analysis of Variance Table

Source	d.f.	s. sq.	m.s.s.	F. Value	$F._{05}$
Doses (adjusted for time)	3	0.0263	0.0087	1.61	3.13
Times (adjusted for doses)	4	0.0356	0.0089	1.64	2.90
Error	19	0.1024	0.0054		
Total	26	0.1770			

APPENDIX 1 C

% Prophases/Basal Cells - Palate.

Time After Injection	Group 1	Group 2	Group 3	Group 4
½ hour	1.19	1.22 1.19		1.48 1.72
1 hour		1.32 1.95	1.27 1.67	1.28 1.42
2 hours	1.59	1.58 1.83	1.66 1.29	
4 hours	1.72	1.75 1.42	1.66 1.46	1.44 1.65
8 hours	1.22	1.80	1.89	1.32 1.56

Analysis of Variance Table

Source	d.f.	s. sq.	m.s.s.	F. Value	$F_{.05}$
Doses (adjusted for time)	3	0.0532	0.0177	0.323	3.07
Time (adjusted for doses)	4	0.1826	0.0456	0.832	2.84
Error	21	1.1525	0.0548		
Total	28	1.4006			

APPENDIX 1 C

% Prophases/Basal Cells - Tongue.

Time After Injection	Group 1	Group 2	Group 3	Group 4
½ hour	1.59	1.80 1.56		1.95 1.59
1 hour		1.37 1.37	2.10 1.55	1.91 2.63
2 hour	1.37	1.46 1.97	2.30 1.57	
4 hour	2.03	2.03 1.90	1.49 2.21	1.71 2.19
8 hour	1.53	1.78 1.66	1.96	1.62 1.81

Analysis of Variance Table

Source	d.f.	s. sq.	m.s.s.	F. Value	$F_{.05}$
Doses (adjusted for time)	3	0.2612	0.0871	0.92	3.07
Time (adjusted for doses)	4	0.2056	0.0514	0.54	2.84
Error	21	1.9853	0.0945		
Total	28	2.5295			

APPENDIX 1 D

Student's t - test for Slopes

$$t = \frac{\text{difference between slopes}}{\text{standard error of difference}}$$

i.e. $$t = \frac{(b_1 - b_2)}{\sqrt{\left(\frac{S^2}{\Sigma x_1^2} + \frac{S^2}{\Sigma x_2^2}\right)}}$$

Where $$S^2 = \frac{S_1^2(n_1 - 1) + S_2^2 (n_2 - 1)}{(n_1 + n_2 - 2)}$$

Cheek Pouch: Gr. 2 and Gr. 4.

Difference between slopes (1.02 - 0.34)	=	0.68
s.e. of difference	=	0.1418
t	=	4.79

$p < .001$

Gr. 3 and Gr. 4.

Difference between slopes (1.02 - 0.79)	=	0.23
s.e. of difference	=	0.1850
t	=	1.243

$p > .20$

Palate: Gr. 2 and Gr. 4.

Difference between slopes (2.99 - 2.32)	=	0.67
s.e. of difference	=	0.3067
t	=	2.185

$p < .05$

APPENDIX 1 D (Contd.)

Palate (contd.) Gr. 3 and Gr. 4.

Difference between slopes (3.09 - 2.99)	=	0.10
s.e. of difference	=	0.4348
t	=	0.230

$p > .8$

Tongue: Gr. 2 and Gr. 4.

Difference between slopes (3.02 - 1.13)	=	1.89
s.e. of difference	=	0.4472
t	=	4.23

$p < .001$

Gr. 3 and Gr. 4.

Difference between slopes (3.02 - 2.57)	=	0.45
s.e. of difference	=	0.6252
t	=	0.28

$p > .7$

APPENDIX 2 A

Cheek Pouch.

% Labelled mitosis/mitosis : % Labelled cells/Basal cells
% Mitosis/Basal cells.

Time	LM	Total M	% $LM/_M$	LC	M	Cells	% $LC/_C$	% $M/_C$
1 hr.	8	171	4.67	210	63	5000	4.20	1.26
2 hrs.	44 76	155 275	28.39 27.63	173 268	51 52	4000 5000	4.32 5.36	1.27 1.04
3 hrs.	53	96	55.21			5000		
4 hrs.	81 40	109 68	74.31 58.82	350 136	48 25	5000 1800	7.00 7.55	0.96 1.38
6 hrs.	94 120	109 132	86.24 90.90	385 316	58 48	5000 4800	7.70 6.58	1.16 1.00
8 hrs.	79	82	96.34	252	43	5000	5.04	0.86
9 hrs.	91	98	92.85	265	44	4200	6.31	1.05
12 hrs.	70	146	60.34	327 153	47 25	4900 2500	6.67 6.12	0.96 1.00
14 hrs.	49 35	112 77	43.75 38.28					
16 hrs.	35	156	22.43					
18 hrs.	29 13	223 128	12.88 10.16					
20 hrs.	2	79	2.53					
22 hrs.	0	73	0.00					
24 hrs.	0	148	0.00					

	$\%LC/_C$	$\%M/_C$
Mean:	6.10	1.08
S.E.:	0.33	0.04

APPENDIX 2 B

Percentage Labelled Mitosis/Mitosis.

Palate.

	Labelled		Non-Labelled		% $LMB/_{BM}$	% $LSBM/_{SBM}$	Overall $\%LM/_{M}$
	Basal	S-basal	Basal	S-basal			
N_1A	2	3	71	48	2.739	5.882	4.032
* N_2A	4	1	11	4	26.67	20.00	25.05
N_2B	12	6	25	10	32.43	37.50	33.96
N_3A	55	30	15	13	78.57	69.76	75.22
* N_4A	22	9	2	1	91.66	90.00	91.18
N_4B	49	16	8	1	85.96	94.11	87.84
N_6A	29	17	5	3	85.29	85.00	85.18
N_6B	39	17	1	0	97.50	100.00	98.24
* N_8A	19	17	3	0	86.36	100.00	92.30
N_9A	45	38	13	6	77.586	86.36	81.37
$N_{12}A$	25	9	75	23	25.00	28.125	25.75
$N_{12}B$	16	7	26	15	38.09	31.81	35.93
* $N_{14}A$	3	2	28	21	9.677	8.696	9.260
$N_{14}B$	3	2	28	13	9.677	13.33	10.86
$N_{16}A$	5	5	51	37	8.928	11.904	10.20
$N_{18}A$	4	2	67	38	5.63	5.000	5.40
$N_{18}B$	1	0	68	44	1.449	0.00	0.8849
$N_{20}A$	3	0	89	35	3.2608	0.00	2.36
$N_{22}A$	1	1	110	52	0.9009	1.88	1.21

APPENDIX 2 B

% Labelled Basal Cells/Basal Cells - Palate.

Time After Injection	Labelled Basal	Labelled Supra-basal	Total Basal	% Labelled Basal
1 hour	303	164	3150	9.62
2 hours	202	91	1860	10.86
3 hours	194	88	2120	9.15
4 hours	258	120	2000	12.90
	285	141	2225	12.81
6 hours	181	110	2000	9.05
	268	166	2000	13.40

Mean = 11.11%

S.Error = 0.72

% Basal Mitosis/Basal Cells - Palate.

Time After Injection	Basal Mitosis	Basal Cells	% Mitosis
1 hour	46	1700	2.70
2 hours	44	1860	2.36
3 hours	49	2120	2.31
4 hours	44	2000	2.20
	37	2225	1.66
6 hours	38	2000	1.90
	44	2000	2.20

Mean = 2.19%

S.Error = 0.12

APPENDIX 2 C.

Significance tests for the differences between the labelling and mitotic indices in the epithelium of the cheek pouch and the palate.

Labelling Index

Palate		Cheek Pouch	
$\bar{x}_1$	= 11.11	$\bar{x}_2$	= 6.10
Variance	= 3.6176	Variance	= 1.3258
n_1	= 7	n_2	= 11

$$\text{Ratio of variances} = \frac{3.6176}{1.3258}$$

$$= 2.729$$

($F_{.05}$, df 6,10 = 3.22)

Hence variances are equal.

Pooled variance (S^2) = 2.269

$$\text{Standard error of difference} = \sqrt{\frac{S^2}{n_1} + \frac{S^2}{n_2}}$$

$$= \sqrt{\frac{2.269}{7} + \frac{2.269}{11}}$$

$$= 0.7213$$

$$t = \frac{(x_1 - x_2)}{\text{Standard error of difference}}$$

$$= \frac{5.01}{0.7213}$$

$$= 6.93$$

$$p < 0.001$$

APPENDIX 2 C (contd.)

Mitotic Index

Palate		Cheek Pouch	
$\bar{x}_1$	= 2.19	$\bar{x}_2$	= 1.08
Variance	= 0.1114	Variance	= 0.0207
n_1	= 7	n_2	= 11

$$\text{Ratio of variances} = \frac{0.1114}{0.0207}$$

$$= 5.38$$

($F_{.05}$, df 6,10 = 3.22)

Hence variances are not equal and Student's t-test cannot be applied.

Fisher-Behrens test.

The difference of means is judged to be significant if it exceeds $d\sqrt{S_1^2 + S_2^2}$, where S_1^2 and $S^2 2$ are the variance of the respective means and d is the value obtained from the tables (Fisher & Yates, 1963) corresponding to n_1, n_2 and θ, where n_1 and n_2 are the number of observations in the two groups and θ is obtained from the formula $\tan \theta = \frac{S_1}{S_2}$.

$$\tan \theta = \sqrt{\frac{0.01591}{0.00188}}$$

$$= 2.91$$

$$\theta = 71^\circ 6'$$

For/

APPENDIX 2 C (contd.)

For the above values of n_1, n_2 and θ, the value of d at the 95% level of confidence is 2.41.

Therefore $d\sqrt{S_1^2 + S_2^2} = 2.41\sqrt{0.01591 + 0.00188}$

$= 0.3232$

$(\bar{x}_1 - \bar{x}_2) = (2.19 - 1.08)$

$= 1.11$

Hence the difference of means is significant.

APPENDIX 3 A

Percentage labelled cells/basal cells in hyperplastic epithelium of animals after single and double injections of tritiated thymidine.

One Injection			Two Injections		
LC	Cells	%LC	LC	Cells	%LC
290	2500	11.60	253	2000	12.65
296	2500	11.84	258	2000	12.90
319	3000	10.63	273	2000	13.65
2333	2000	11.65	264	2000	13.200
331	3000	11.03	254	2000	12.70
194	1900	10.21	262	2000	13.10
219	2000	10.95	265	2000	13.25
203	2000	10.15	250	2000	12.50

$\bar{X}_1$ = 11.01

Variance = 0.4249

s. error = 0.23

$\bar{X}_2$ = 12.99

Variance = 0.1439

s. error = 0.13

APPENDIX 3 B.

Percentage arrested metaphases/basal cells in hyperplastic cheek pouch epithelium.

Time after injection of vinblastine	Metaphases	Basal Cells	% Metaphases/ Basal Cells
2 hrs.	110	4000	2.75
	99	3000	3.30
	91	3000	3.03
	109	3000	3.63
	101	3000	3.36
Mean			3.21
S.Error			0.15
6 hrs.	246	4000	6.15
	209	3000	6.96
	244	3000	8.13
	220	3000	7.33
	270	3000	9.00
Mean			7.51
S.Error			0.48

APPENDIX 3 B.

Percentage mitosis/basal cells in hyperplastic cheek pouch epithelium.

	Hyperplastic		
	Mitosis	Cells	% Mitosis
1	59	3000	1.96
2	79	3000	2.63
3	56	3000	1.86
4	51	3000	1.70
Mean			2.04
S.Error			0.20

APPENDIX 3 C.

$$\text{Slope (b)} = \frac{\Sigma xy}{\Sigma x^2}$$

$$s^2 = \frac{(y^2 - b \cdot \Sigma xy)}{(n - 1)}$$

$$\text{Standard error of (b)} = \sqrt{\frac{s^2}{\Sigma x^2}}$$

The values of Σxy, Σx^2 and Σy^2 were computed from the data on the percentage arrested metaphases (Appendix 3 B) and were found to be:-

$$\Sigma xy = 257.56$$

$$\Sigma x^2 = 200$$

$$\Sigma y^2 = 339.19$$

$$b = \frac{257.56}{200}$$

$$= 1.29$$

$$s^2 = \frac{(339.19 - 332.20)}{9}$$

$$= 0.7567$$

$$\text{Standard error of (b)} = \sqrt{\frac{0.7567}{200}}$$

$$= 0.06$$

APPENDIX 3 D.

Significance tests for the mitotic and labelling indices of normal and hyperplastic epithelia.

Since the variances in the two groups are not equal, student's t-test is not applicable and the differences have to be tested by Fisher-Behren's test. The difference between means is judged to be significant if it exceeds $d\sqrt{S_1^2 + S_2^2}$ where S_1^2 and S_2^2 are the variances of the respective means and d is the value obtained from the tables (Fisher & Yates, 1963) corresponding to n_1, n_2 and θ where n_1 and n_2 are the number of observations in the two groups and θ is obtained from the formula : $\tan \theta = \frac{S_1}{S_2}$.

Labelling Indices

Hyperplastic Epithelium	Normal Epithelium
$\bar{x}_2 = 9.17$	$\bar{x}_1 = 6.10$
Variance = 0.2950	Variance = 1.325
$n_2 = 8$	$n_1 = 11$
$S_2^2 = 0.0368$	$S_1^2 = 0.1204$

$$\tan \theta = \sqrt{\frac{0.1204}{0.0368}}$$

$$= 3.185$$

$$\theta = 72^\circ\ 33^1$$

For the values of n_1, n_2 and θ, the value of d at the 95% level is 2.18

$$d\sqrt{S_1^2 + S_2^2} = 2.18\sqrt{0.1204 + 0.0368}$$

$$= 0.1475$$

$$(\bar{x}_2 \doteq \bar{x}_1) = 3.07$$

Hence the difference of means is significant.

APPENDIX 3 D (contd.)

Mitotic Indices

Hyperplastic Epithelium	Normal Epithelium
$\bar{x}_1 = 1.70$	$\bar{x}_2 = 1.08$
Variance = 0.1163	Variance = 0.0160
$n_1 = 4$	$n_2 = 11$
$S_1^2 = 0.0291$	$S_2^2 = 0.00145$

$$\tan \theta = \frac{0.0291}{0.00145}$$

$$= 200.4$$

$$\theta = 89^{\circ}\ 42^{1}$$

d 0.05 (from tables) = 2.45

$$d\sqrt{S_1^2 + S_2^2} = 2.45\ \sqrt{0.0291 + 0.00145}$$

$$= 0.4283$$

$$(\bar{x}_1 - \bar{x}_2) = 0.62$$

Hence the difference of means is significant.

APPENDIX 4 A.

Significance of the difference between the latent period for the induction tumours in golden hamsters, and albino and spotted hamsters.

Golden hamsters		Albino and spotted Hamsters	
$\bar{x}_1$	= 9.34	$\bar{x}_2$	= 10.77
Variance	= 3.35	Variance	= 0.719
n_1	= 27	n_2	= 9

$$\text{Ratio of variances} = \frac{3.35}{0.719}$$

$$= 4.66$$

$$(F_{.05} \text{ df } 26,8 = 3.12)$$

Hence variances are not equal, and Student's t-test is not applicable.

Fisher-Behrens test.

The difference between means is judged to be significant if it exceeds $d\sqrt{S_1^2 + S_2^2}$ where S_1^2 and S_2^2 are the variances of the respective means and d is the value obtained from the tables (Fisher & Yates, 1963) corresponding n_1, n_2 and θ, where n_1 and n_2 are the number of observations in the two groups and θ is obtained from the formula $\tan \theta = \frac{S_1}{S_2}$.

$$\tan \theta = \frac{0.1241}{0.0799}$$

$$= 1.552$$

$$\text{Therefore } \theta = 57^\circ 12'$$

For/

APPENDIX 4 A (contd.)

For the above values of n_1, n_2 and θ the value of d at the 95% level of confidence is 2.06.

Therefore $d\sqrt{S_1^2 + S_2^2} = 2.06\sqrt{0.1241 + 0.0799}$

$= 0.9305$

Difference between means $= (10.77 - 9.34)$

$= 1.43$

Hence the difference of means is significant.

APPENDIX 4 B.

% Labelled mitosis/mitosis; % Labelled cells/cells;
% Mitosis/cells - Squamous cell carcinoma of the hamster cheek pouch.

Time hrs.	LM	Total M	%LM/M	LC	M	Cells	%LC	%M
½	2	63	3.17	424	41	2000	21.20	2.05
	0	96	0.00	388	33	2000	19.40	1.65
2	44	117	57.26	432	42	2000	21.60	2.10
3	86	104	82.62	438	37	2000	21.90	1.85
4	100	104	96.07	342	32	1500	22.68	2.13
	94	96	97.92	441	43	2000	22.05	2.15
5	159	189	84.12	462	47	2000	23.10	2.35
6	96	120	80.00					
7	68	88	77.27					
8	64	127	50.40					
9	32	110	29.09					
10	38	201	16.42					
11	13	184	7.06					
12	23	269	8.55					
14	16	88	18.18					
15	30	116	28.86					
16	54	144	37.50					
17	86	201	43.00					
18	47	72	65.27					
19	70	105	66.66					
20	95	120	79.16					
22	77	103	74.75					
23	64	121	52.89					
24	29	105	20.76					
25	23	105	20.19					
26	12	102	11.76					
						Mean	21.70	2.04
						Standard error	0.4543	0.0856

APPENDIX 4 C.

Percentage arrested metaphases/tumour cells in squamous cell carcinoma of the hamster cheek pouch.

Time after injection of Vinblastine	Metaphases	Tumour Cells	% Metaphases/ Tumour Cells
2 hours	85	2000	4.25
	87	2000	4.35
	95	2000	4.75
5 hours	264	2000	13.20
	284	2000	14.20
	256	2000	12.80

APPENDIX 4 D.

$$\text{Slope (b)} = \frac{\Sigma xy}{\Sigma x^2}$$

$$s^2 = \frac{(\Sigma y^2 - b \cdot \Sigma xy)}{(n-1)}$$

$$\text{Standard error of b} = \sqrt{\frac{s^2}{\Sigma x^2}}$$

The values Σxy, Σx^2 and Σy^2 were computed from the values shown in Appendix 4 C and were found to be:-

$$\Sigma xy = 227.70$$

$$\Sigma x^2 = 87.00$$

$$\Sigma y^2 = 599.27$$

$$b = \frac{227.70}{87}$$

$$= 2.62$$

$$s^2 = \frac{(599.27 - 596)}{5}$$

$$= 0.654$$

$$\text{Standard error of b} = \sqrt{\frac{0.654}{87}}$$

$$= 0.09$$

APPENDIX 4 E.

$$N_t = N_0 \cdot (\alpha)^{t/T_C} \text{ ---------- (4)} \qquad \text{(page 109)}$$

At the instance when $N_t = 2 N_0$, t would be equal to the doubling time of the population.

Thus, $2 N_0 = N_0 \cdot (\alpha)^{t/T_C}$

i.e. $2 = (\alpha)^{t/T_C}$

Converting both sides to logarithmic scale:

$$\log_e 2 = \frac{t}{T_C} \cdot \log_e \alpha$$

Therefore, $t = T_C \cdot \frac{\log_e 2}{\log_e \alpha}$

Since $\alpha = (1 + p)$ (page 109); $t = T_C \cdot \frac{\log_e 2}{\log_e (1+p)}$ ----(5)

APPENDIX 5.

% Labelled mitosis/mitosis - Pre-neoplastic hyperplasia of the hamster cheek pouch.

Time after injection	LM	Total M	%LM/M
½ hr.	4	111	3.60
1	13	112	11.61
2	24	73	32.88
3	71	99	71.73
4	74	89	83.14
5	70	77	90.90
6	123	129	95.35
7	95	104	91.35
8	97	110	88.18
9	73	98	74.49
10	57	95	60.10
11	29	107	27.10
12	18	74	24.32
13	17	73	23.29
14	20	108	18.52
15	10	46	21.74
16	9	104	8.65
17	0	69	0.00
18	23	112	20.53
19	13	69	18.84
20	29	107	27.23
21	18	92	19.56
23	39	126	30.95
24	46	104	44.24
25	44	82	53.44
26	50	90	55.55

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